Detection and Enumeration of Faecal Indicator Bacteria in Water

Puja Tandon

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ABSTRACT

This study investigated the effects of various factors, namely exposure to brass/copper, sunlight, high temperature, chlorine, low pH, or starvation, on the enumeration of faecal indicator bacteria in water, performed using non-selective & selective media under conventional aerobic conditions & under conditions designed to neutralise reactive oxygen species e.g. by the addition of 0.05% w/v sodium pyruvate as a scavenger of peroxides &/or anaerobic incubation, to encourage fermentative metabolism. These methods were compared with standard US & UK methods for the recovery of injured bacteria. The information gained was used for the development of a novel broth-based medium for the enumeration of sub-lethally injured *Escherichia coli* under field conditions. The novel broth-based field medium ('Coliblack') was tested against a currently used method (H_2S test) & evaluated under field conditions with unskilled personnel in rural locations in India.

The results showed that growth conditions designed to neutralise reactive oxygen species could enhance the colony count of faecal indicator bacteria, whether enumerated using a non-selective medium, a selective medium, a resuscitative medium, or a chromogenic growth medium, following exposure to stressors such as storage of water in a brass container, sunlight, high temperature & chlorine. Such conditions may induce sub-lethal injury in *E. coli* & *E. faecalis*, inactivating the bacterial cells under conventional aerobic enumeration conditions as a result of their oxygen-sensitivity. However, exposure to some other stressors namely low pH & starvation was not strongly oxygen-sensitive, as no substantial differences between counts were observed on various growth media or enumeration conditions. The results from enumeration experiments were used to develop a field-based broth medium with a peroxide-neutralising resuscitative agent (sodium pyruvate), a selective agent showing minimum inhibition (Tergitol 7) & a chromogenic diagnostic agent based on defined substrate technology (8 hydroxyquinoline-glucuronide).

The Coliblack medium indicates the presence of *Escherichia coli* in the drinking water by a colour change of the medium to black. Furthermore, the preliminary trial carried out for the evaluation of the novel broth-based field medium gave positive feedback from users. In this respect the results obtained in the present study represents an advancement in understanding of how to maximise the enumeration of faecal indicator bacteria in water & how to apply this information to improve detection under field conditions.

Table of Contents

List of Tables

List of Figures

List of Plates

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Declaration

This thesis records the results of experiments conducted by myself in the School of Applied Sciences, Northumbria University under the supervision of Prof. R.H. Reed and Department of Microbiology, Panjab University, Chandigarh, India under second supervision of Prof. Sanjay Chhibber between May 2002 and November 2005.

This work is of my own composition and has not been previously submitted in part, or in whole, for a higher degree.

Abbreviations and terms

8-HQ-GLUC	8-hydroxyquinoline-β-glucuronide
AC	anaerobic cabinet
ACpre	pre-reduced medium in an anaerobic cabinet
aer	aerobic
AJ	anaerobic jar
BAA	bile aesculin agar
BGBLB	brilliant green bile lactose broth
CC	chromocult agar
CFU	colony forming units
DTT	dithiothreitol
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
ELISA	enzyme linked immunosorbent assay
EMB	eosin methylene blue agar
FAC	ferric ammonium citrate
FAS	ferric ammonium sulphate
FC	faecal coliforms
H_2O_2	hydrogen peroxide
IEA	immuno enzyme assay
IFA	immunofluorescence assay
IMS	immunomagnetic seperation
IMViC	indole, methylred, Voges-Proskauer, citrate tests
ISH	*In situ* hybridization
KF	KF streptococcus agar
LTB	lauryl tryptose broth

LTLB	lauryl tryptose lactose broth
M2	MacConkey number 2
Mac	MacConkey agar
magenta-GLUC	5-bromo-6-chloro-3-indolyl-β-glucuronide
MF	membrane filtration
mFC-R	m-faecal coliform agar without rosolic acid
MG	minerals modified glutamate medium
mLSA	m-lauryl sulphate agar
MPN	most probable number
MU-GAL	4-methylumbelliferyl-β-galactoside
MU-GLU	4-methylumbelliferyl-β-glucoside
MU-GLUC	4-methylumbelliferyl-β-glucuronide
N	nutrient agar
NB	nutrient broth/agar, bile salts
NCTC	National Collection of Type Cultures
NDTT	nutrient broth/agar, dithiothreitol
NP	nutrient broth/agar, sodium pyruvate
NS	nutrient broth/agar, sodium lauryl sulphate
NT	nutrient broth/agar, tergitol 7
NTDTT	nutrient broth/agar, tergitol 7, dithiothreitol
NTP	nutrient broth/agar, tergitol 7, sodium pyruvate
NTX	nutrient broth/agar, tergitol 7, X-GLUC
$O_2^{-\cdot}$	superoxide free radical
ONPG	*ortho*-nitrophenyl-β-galactopyranoside
P/A	presence/absence test
PCR	polymerase chain reaction

peroxide-neutralised conditions	pyruvate-supplemented growth medium incubated aerobically
PUCC	Panjab University Culture Collection
ROS	reactive oxygen species
ROS-neutralised conditions	pyruvate-supplemented growth medium incubated anaerobically
SB	Slanetz and Bartley agar
SOD	superoxide dismutase
TC	total coliforms
TTM	tryptose, tergitol 7, MU-GLUC
TTX	tryptose, tergitol 7, X-GLUC
VBNC	viable but non culturable cells
X-GLUC	5-bromo-4-chloro-3-indolyl-β-glucuronide

Chapter 1

Introduction and literature review

1.1 Background

"Water, water everywhere, nor any drop to drink"- this famous line from the Rime of the Ancient Mariner, written by Samuel Taylor Coleridge, is highly relevant to the global provision of drinking water. Although two thirds of the Earth is covered by water, though only a small fraction is fresh water. Such water is a source provided freely by nature and should be regarded as a social/common resource. When converted into any utilizable form, for example drinking water, it becomes simultaneously an economic good, a social good, a political good, and its usage has strong ethical implications (Agarwal and Narain, 1997). Across the globe, more than 1.2 billion people do not have access to safe drinking water, while others are exposed to significant risks to health because of contamination during transport or storage (WHO, 2002a; Dieterich, 2003; WHO, 2004a; Kerwick *et al.*, 2005). Despite major efforts to deliver safe, piped, community water to the world's population over the past two decades, the reality is that such water supplies will not be available to all people for the forseeable future (Mintz *et al.*, 2001). The alarming rate of increase in the world's population has caused the situation to deteriorate further and it has been calculated that by 2025, one third of the World's population may suffer from chronic water shortage (Percival *et al.*, 2000; WHO, 2004b). Remedies to control such situations have been set up by the United Nations in 2000 by establishing the Millennium Declaration to decrease by half the people without access to safe drinking water by 2015 (WHO, 2004c; Anon., 2005). The United Nations General Assembly on World Water Day 2005, declared that the years from 2005 to 2015 would be committed to achieving several key international goals related to water such as access to clean water for sanitation and health, integrated water resources management, trans-boundary water issues and many more proclaiming it to be the decade of "Water for Life" (Pittet, 2005; UNICEF, 2005).

In the Indian context, studies have shown that almost three-quarters of all available drinking water sources are polluted with faecal bacteria and over 73 million workdays are estimated to be lost due to diseases related to water (Agarwal, 1981; Agarwal and Narain, 1997; Patil *et al.,* 2002; Reed *et al.*, 2005). Such diseases constitute a major burden to health: diarrhoea alone is a cause of up to 2.5 million deaths among children aged below five in developing countries (Kosek *et al.,* 2003; MacDonald, 2004; Thapar and Sanderson, 2004) and up to 88% of infectious diseases worldwide (Kindhauser, 2003). Cholera (profuse watery diarrhoea) and dysentery (severe diarrhoea with blood in the stools) are life-threatening forms of diarrhoea (WHO; 2004a).

1.2 Diseases linked to the water supply

Diseases caused by contaminated water supplies can be divided into four groups (modified from Ashbolt, 2004):

a) ***Water-borne diseases***: the route for transmission is water, which acts as a passive carrier for infectious pathogens. Poor sanitation can also be another cause. The diseases included under the water-borne category include cholera, typhoid, bacillary dysentery, infectious hepatitis, leptospirosis, giardiasis and gastroenteritis. Improving the quality of water and sanitation can help in overcoming these diseases.

b) ***Water-related diseases***: the route for transmission of these diseases is via vectors such as mosquitoes, flies and insects, which live near or close to sources of water. The diseases included in this group include yellow fever, dengue fever, encephalitis and sleeping sickness.

c) ***Water-based diseases***: these diseases are caused by infecting agents that are spread by contact with, or ingestion of, water as the latter supports an essential part of the life cycle of the former e.g. aquatic snails. The diseases included in this group include schistosomiasis, dracunculosis, bilharziosis, threadworm and other helminth infections.

d) ***Water-washed diseases***: these diseases are caused by lack of water for maintenance of personal hygiene and sanitation. The diseases included in this group are scabies, trachoma, leprosy, conjunctivitis, salmonellosis, ascariasis, trichuriasis, hookworm and paratyphoid fever.

1.3 Water sources and treatments

The major sources of drinking water are either surface waters, such as rivers and lakes, lowland reservoirs and spring water, or ground water sources, including hand pumps and wells. To obtain wholesome drinking water meeting the requirements of microbiological and chemical quality, water is often treated. The treatment methods can be divided into physical methods and chemical methods, which include water treatments carried out at the municipal or community level as well as those at the household level (point-of-use water treatment systems), as categorized by the World Health Organization (2002a).

For an individual family, the physical methods include:

(a) ***Boiling or heating with fuel***: this method to treat water has been used since ancient times and is effective in destroying water-borne pathogens such as viruses, bacteria and some bacterial spores, fungi, protozoan cysts and helminth ova (USEPA, 1993; Nieuwoudt and Mathews, 2005). This treatment can be easily applied to waters with high turbidity and dissolved constituents. It is recommended to boil water for at least 15 minutes

whereas heating at 55°C for several hours (pasteurization) has been reported to reduce non-spore forming bacterial pathogens as well as many viruses and parasites, including the water-borne protozoa *Cryptosporidium parvum*, *Giardia lamblia* and *Entamoeba histolytica* (Feachem *et al.,* 1983; Sobsey and Leland, 2001).

(b) ***Thermal treatment with solar radiation and solar cooking:*** this approach using sunlight to heat involves water at lower temperatures than boiling, using solar radiation for longer periods, termed as solar pasteurization (Burch and Thomas, 1998; Nieuwoudt and Mathews, 2005). In India this treatment has been practised since 2000 B.C (Patwardhan, 1990; Reed *et al.*, 2005). Solar pasteurization of water, other beverages and foods for young infants is a practical, accessible and affordable option at the household level. This approach is feasible as low cost solar reflectors or cookers can be made from materials as simple and economical as cardboard and aluminum foil (Rijal and Fujioka, 2003; Negi and Purohit, 2005). This technology for water treatment and food preparation has been field tested in many parts of the world, including Kenya, Tanzania, Ethiopia, Vietnam and some developing countries in South America (WHO 2002a). It is effective against most enteric viruses, bacteria and parasites (Ciochetti and Metcalf, 1984).

(c) ***Solar disinfection by the combined action of heat and UV radiation*:** this method of treatment of water inactivates water-borne pathogens during exposure of contaminated water to sunlight in clear vessels as a result of the combined effects of UV-A radiation (320-400 nm) and heat (50°C -60°C). It has been studied and applied in field work by many researchers (e.g. Acra *et al.,* 1984; Wegelin *et al.*, 1994; Conroy *et al.*,

1996; Joyce *et al.*, 1996; Reed, 1997; Wegelin and Sommer, 1998; Reed, 2004; Lonnen *et al.*, 2005) and has been named SODIS (solar disinfection). It has been noted that water can be heated to temperatures of 55°C in transparent bottles (e.g., clear plastic bottles, Reed, 2004) exposed to sunlight for several hours. Researchers such as Wegelin *et al.* (1994) and Joyce *et al.* (1996) have reported an increase in the thermal effect when the bottles are painted black on one side or are lying on a dark surface that collects and radiates heat (Wegelin *et al.*, 1994; Joyce *et al.*, 1996). However, UV-A radiation in sunlight as well as the thermal effects of solar radiation also inactivates water-borne microbes e.g. Acra *et al.* (1984) reported that a 95 minute exposure to bright sunlight is effective in inactivating 99.9% of faecal coliforms, while 300 minutes can inactivate all bacteria (Kehoe and Conroy, 2002). This treatment is effective against faecal coliforms, including *Escherichia coli* (*E. coli*), and enterococci such as *Enterococcus faecalis* and viruses such as coliphage F2, rotavirus and encephalomyocarditis (EMC) virus (Khaengraeng, 2004; Martín-Domínguez *et al.*, 2005).

(d) ***UV disinfection with a germicidal lamp:*** the germicidal property of UV radiation from lamps was recognized in the late 1800s, whereas its utility for the disinfection of drinking water was introduced only in the 20th Century (Blatchley and Peel, 2001). The wavelengths of germicidal UV lamps lie between 200 nm and 320 nm and they use medium pressure, temperatures and power levels to produce radiation that directly damages DNA (UV-C radiation, ≈ 265 nm) to permanently inactivate the target microbes. This method has been effective against water-borne chlorine-

resistant protozoa including *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts (WHO 2002a; Jin *et al.*, 2005).

(e) ***Water storage in an appropriate vessel:*** this is one type of household point-of-use remedy where a narrow-mouthed container with a tap and a close-fitted lid such as a water urn (Chidavaenzi *et al.*, 1998) or an earthernware vessel or "zir" (Mintz *et al.*, 1995) is used for collecting water from a source. Such narrow mouth vessels with proper taps and tight-fitted lids minimise the risk of post-collection contamination (Wright *et al.*, 2004). When combined with point-of-use chlorination (see later) this simple and low-cost remedy is within the limits of many households and has been effective in reducing diseases caused by water-borne pathogens by >90%, e.g. water-borne diseases such as cholera were reduced by 17-90% by such home treatments in studies in Bolivia and Zambia (Quick *et al.*, 1996; Quick *et al.*, 1999), and faecal and total coliforms present in water by 50% (Chidavaenzi *et al.*, 1998; Mazengia *et al.*, 2002).

(f) ***Filtration:*** Filtration is another traditional physical method that is widely used in some locations and removes particles and at least some microbes from water. This can be done using different types of filters such as granular rapid filters (sand, gravel, diatomaceous earth, coal and other minerals), ceramic and other porous cast filters (clay and other minerals), slow sand filters and septum-body feed filters such as diatomaceous earth (AWWA, 1997; WHO, 2002a). These are effective in removing 90-99% of viruses and bacteria (LeChevallier and Au, 2000; Saito and El-Ghetany, 2002).

(g) **Coagulation- flocculation and sedimentation**: Coagulation is often a primary step in conventional large-scale water treatment and involves the gathering together of fine particles to form clumps (Geldreich and Reasoner, 1990; Pokhrel and Viraraghavan, 2004). It requires optimum dose, pH and mixing conditions. Aluminium, iron, and calcium salts are chemical flocculants that are commonly used. In household-level water treatment people use seeds of *Strychnos potatorum* (Nirmali plant), *Moringa oleifera* (horseradish tree), and bentonite clay of *Rauwaq* (Gupta and Chaudhuri, 1992; Kehoe and Conroy, 2002). Sedimentation can be carried out at the household level by storage of water in large pots or buckets (Partwardan, 1990). Basically, at least two containers will be needed to settle the water: one to act as the settling vessel and another to be the recipient of the supernatant water after the settling period. Usually sedimentation is enhanced by addition of alum, which forms a sticky flocculant and is precipitated (Spellman, 2003). The World Health Organisation has recommended a three pot water storage method for emergency treatment in which 50% of pathogens die during storage and most of the remainder settle out during storage as a point-of-use method (Kayaga, 2005).

(h) ***Combined systems of flocculation and chlorine disinfection:*** this type of treatment is partly physical and partly chemical and can be used in municipal water purification systems and also at home. It is effective for surface waters of high turbidity (Crump *et al.,* 2004). The household-level flocculant-disinfectant technology "PUR" (Procter and Gamble) consists of a powder, which contains ferric sulfate, bentonite, sodium carbonate, chitosan, polyacrylamide, potassium permanganate, and calcium

hypochlorite in a small sachet (Reller *et al.,* 2003) and is effective in rapidly disinfecting moderate amounts of water (e.g. 10 L). The "PUR" water treatment occurs by the combined effects of precipitation, coagulation, and flocculation with disinfection. This facilitates the removal of suspended organic matter, bacteria, viruses, parasites, and heavy metals from the treated water (Souter *et al.*, 2003).

The small-scale chemical disinfection methods include:

(a) ***Chlorine-based compounds****:* these are often used as a final step in conventional water treatment, involving chlorine or one of its derivatives, e.g. hypochlorite, chloramine (chlorine combined with ammonia or organic amines), chlorine dioxide, gaseous chlorine, etc. Chlorine reacts with water to form hypochlorous acid which dissociates to produce the hypochlorite ion along with a hydrogen ion which thereby decreases the pH of water and the hypochlorite ion is highly reactive at relatively low concentrations, thereby inactivating many water-borne pathogens with the exception of cysts of protozoa, such as *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts (Mintz *et al.,* 1995; Reller *et al.,* 2003). For example, free chlorine in a dosage of 0.1-0.3 mg L^{-1} for 30 minutes can be effective in inactivating >99.99% of enteric bacteria and viruses (Laplace *et al.*, 1997). However, the disadvantages of chlorination such as unfavourable taste and odour, occasional failures against resistant micro-organisms, and the generation of potentially toxic disinfection by-products is pressurising the water supply industry to find substitutes (Driedger *et al.*, 2000; Bull *et al.*, 2001; Hallam *et al.*, 2002; Kerwick *et al.*, 2005).

(b) ***Ozone:*** the use of ozone as a drinking water disinfectant has been practised since the 1920s. It has gained popularity for community water supplies in developed countries because it is a strong oxidant, capable of rapidly and extensively inactivating a variety of water-borne pathogens, including chlorine-resistant *Cryptosporidium parvum* oocysts (WHO 2002a; Jyoti and Pandit, 2003). Water ozonizers (Choudary and Reddy, 1995) are used at the household level to generate treated water for general household activities, disinfecting micro-organisms, removing metals and chlorine from water, etc (Ngo and Sahgal, 1989). The effectiveness of water ozonators can be enhanced by the use of catalysts such as iron or iron oxides or other metals for the oxidation of water pollutants (Pines and Reckhow, 2002; Beltrán *et al.*, 2005).

(c) ***Other agents***: iodine, silver, copper, quaternary ammonium compounds and some other chemical agents and disinfectants have been used for the inactivation of water-borne pathogens (Kerwick *et al.*, 2005). However, these chemical agents are often considered as unsuitable for long-term drinking water disinfection (WHO 2002a). Furthermore, the selection of the most appropriate chemical compound and its dose depends on microbial water quality targets or performance targets and problems of intermittent supply and inconsistent quality may occur in rural commodities in developing countries (Percival *et al.*, 2004; WHO, 2004a).

1.4 Microbial indicators of water quality and their characteristics

Satisfactory water quality is defined operationally as the total absence of disease-producing microbes or indicator micro-organisms in 100 mL water samples following disinfection or treatment (Clesceri *et al.*, 1998; Pillai *et al.*, 1999; Kehoe and Conroy, 2002; Bharath *et al.*, 2003). The indicator micro-organism is used to check or measure the efficiency of water treatment as well as any post-treatment contamination or deterioration. Microbiological indicators have been used since the early days of water microbiology, and they have also been employed for the assessment of faecal pollution of water sources. In the strictest sense, the term "indicator" should be used to measure post-treatment efficiency while the term "index" should be used as a quantitative measure of faecal pollution (Waite, 1991; WHO, 2004a). At a practical level, the scientific community has based the definition of microbial faecal indicators on criteria where the same organism would serve both as an indicator and as an index of water pollution. An ideal indicator is defined as possessing the following characteristics (Kehoe and Conroy, 2002; WHO 2004a):

- it should be present in polluted or faecally contaminated water, demonstrating the possible presence of enteric pathogens;
- it should not be capable of multiplying in aquatic habitats;
- it should respond to water treatments or disinfection and environmental stresses in a manner similar to pathogens of concern;
- it should be easily isolated, identified and enumerated on culture media;
- the method used for isolating and enumerating should be economical;
- the density of its presence should be directly correlated with the degree of faecal pollution;
- it should be present in numbers higher than other pathogens;
- it should be non-pathogenic.

Since the earliest microbiological investigations of safe and hygienic drinking water quality, post-treatment efficiency and point-of-use contamination, the presence or absence of faecal indicator bacteria e.g. thermotolerant (faecal) coliforms or faecal streptococci (enterococci) in water have been used to measure faecal pollution (Anon., 2002; WHO, 2004a). For example, the faecal coliform group consists of bacteria with well-defined biochemical and growth characters that are readily identified as a means of monitoring faecal contamination.

1.4.1 Characteristics of the coliform group

The coliform group consists of bacteria which are members of the family Enterobacteriaceae, are capable of multiplying at 37°C and possess β-galactosidase activity (Clesceri *et al.*, 1998; Anon., 2002). They are used to provide basic water quality information from a source but they are not a specific index of faecal pollution (WHO, 2004a). They are Gram-negative, oxidase-negative, lactose-fermenting, non-spore-forming rod-shaped bacteria capable of growth in the presence of bile salts or other surface-active agents with similar growth-inhibiting properties (Clesceri *et al.*, 1998; Anon., 2002). The bacteria in this group include *E. coli, Citrobacter freundii, Enterobacter cloacae,* and *Klebsiella* spp (Figure 1.1).

The faecal or thermotolerant coliforms are bacteria capable of growth at higher temperatures, such as 44°C in the UK and 44.5°C in the US, fermenting lactose at this temperature and are of faecal origin e.g. *E. coli*, which ferments lactose and mannitol with the production of acid and gas at 44-45°C with the exception of a few strains which grow only at 37°C (Clesceri *et al.*, 1998; Anon., 2002; Percival *et al.*, 2004; WHO, 2004a), produces indole from tryptophan, contains β- galactosidase and β-glucuronidase enzymes, is catalase-positive, oxidase-negative and urease-negative,

gives a positive result in the methyl-red test and a negative result in the Voges-Proskauer test (Figure 1.1). *E. coli* is effective both as an indicator and an index of faecal pollution since it is abundant in the faeces of animals and humans and is predominantly found in raw sewage, treated effluents, and all natural waters and soils (Clesceri *et al.*, 1998; Anon., 2002; Khaengraeng, 2004). Additionally, it can be isolated, identified and enumerated on routinely used inexpensive culture media (Manafi, 2000). Its presence in a treated water supply suggests failure in disinfection or the entry of contaminated water (Calabrese and Bissonnette, 1990a; LeChevallier and Au, 2000). While most strains of *E. coli* are non-pathogenic, there are some pathogenic strains causing enteric illness such as enteropathogenic, enteroinvasive, enterotoxigenic and enterohaemorrhagic strains (Bopp *et al.*, 1999; Percival *et al.*, 2004).

1.4.2 Characteristics of the faecal streptococci or enterococci

These bacteria are Gram-positive chain-forming cocci (streptococci) and faecal in origin. In UK they are taken as secondary indicators of faecal pollution (Anon, 2002). They are grouped into Lancefield group D within the streptococci. *Enterococcus* spp. (enterococci) are the major sub-group of the faecal streptococci having a tolerance to sodium chloride, bile salts, sodium azide and to alkaline pH (Reuter, 1995; Anon, 2002). These bacteria are also used for indexing human faecal pollution and for assessing water treatment efficiency. They are often claimed to be more resistant to stress and chlorination than *E. coli* and other coliform bacteria (Flahaut *et al.*, 1996; Laplace *et al.*, 1997). See Figure 1.2.

The ratio of counts of thermotolerant coliforms and faecal streptococci is sometimes used for differentiating contamination of sources from human and animals where a

ratio higher than four indicates contamination from human faeces and less than 0.7 indicates contamination from animal faeces (Clesceri *et al.*, 1998; WHO, 2004a).

Figure 1.1 Flow chart for the differentiation of total coliforms

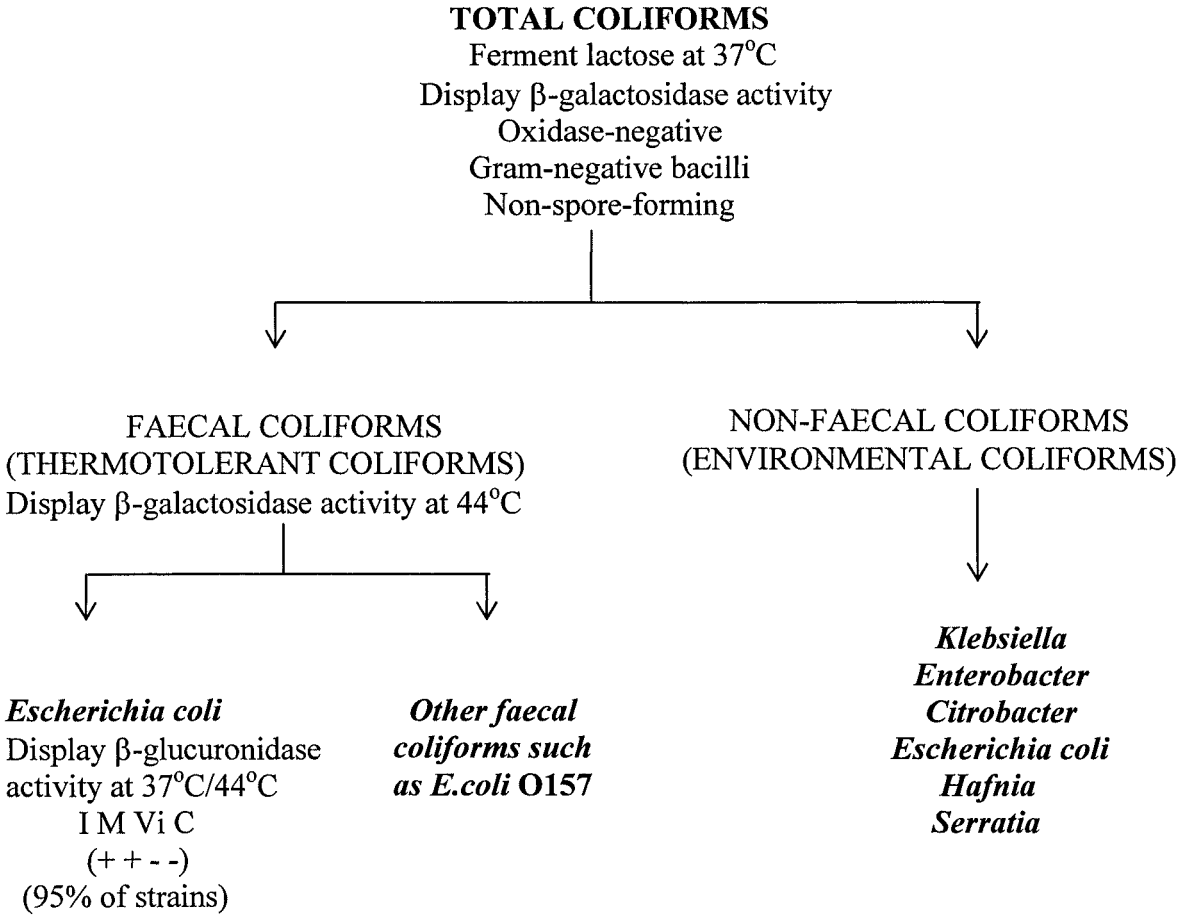
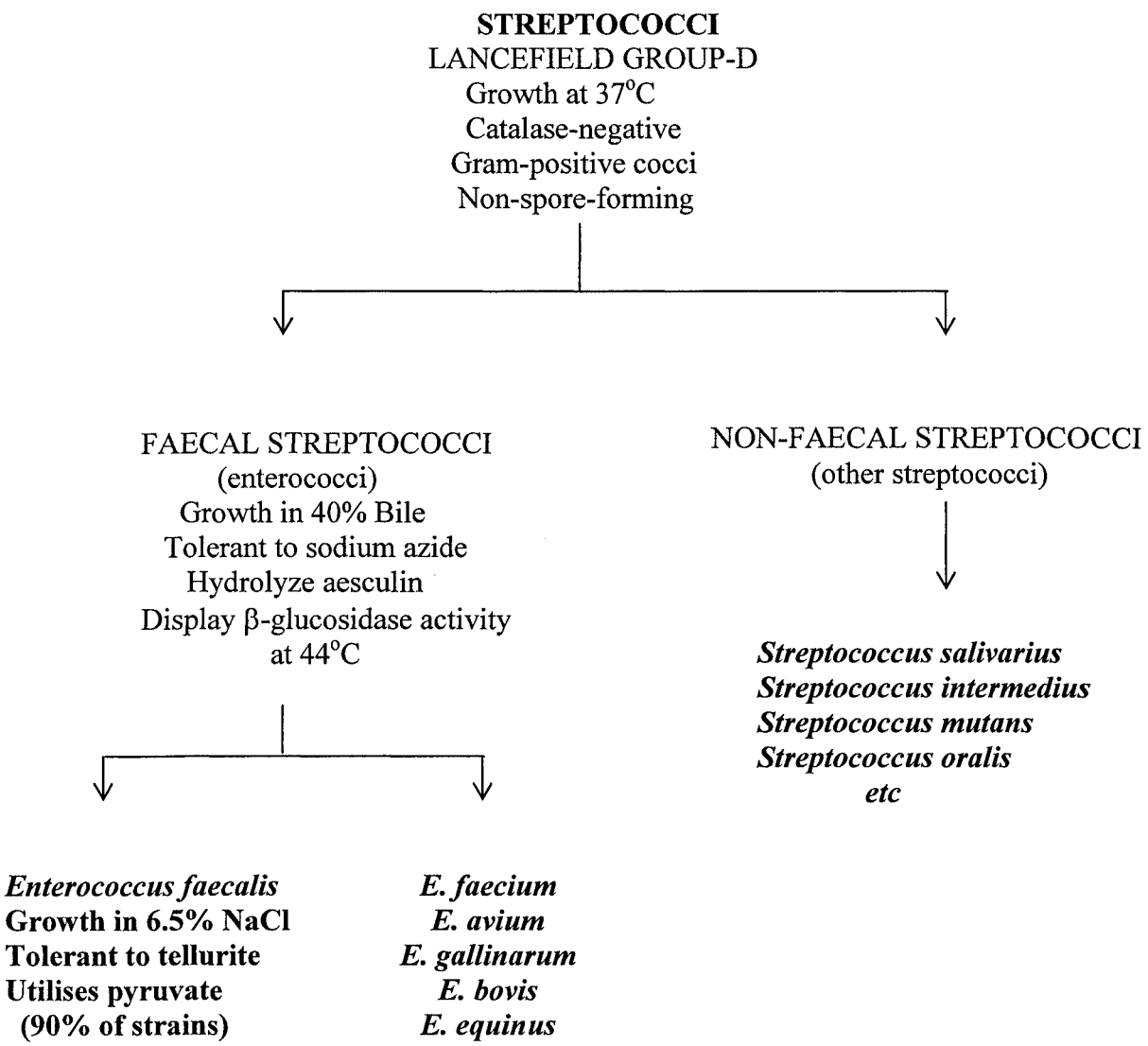


Figure 1.2 Flow chart for the differentiation of streptococci group



1.5 Guidelines for microbiological quality of drinking water

This approach has been used throughout the twentieth century as an important means of preventing water-borne diseases. The World Health Organisation first proposed guidelines for drinking water quality in 1984, which have been revised periodically since then (e.g. WHO, 2004a). The commonest microbiological indicators used in such guidelines are faecal coliforms as shown in Table 1.1 (Vasudevan and Pathak,

1998; Barrel *et al.*, 2000; Vasudevan *et al.,* 2001; WHO, 2004a). According to the guidelines there should be a total absence of faecal bacteria in 100mL of drinking water supplied to consumers after treatment. The United States European Protection Agency (EPA) has introduced the concept of the "Total Coliform Rule" according to which a maximum contaminant level (MCL) goal for total coliforms (faecal coliforms and other coliforms) in 100 mL of drinking water sample is taken as zero (EPA, 1989). The EU Drinking Water Directive was launched in 1989 with the enforcement of the water quality regulations (Gleeson and Gary, 1996). The directive included microbiological parameters for total coliforms, faecal coliforms, enterococci, sulphite reducing anaerobes and colony counts at 22°C and 37°C. Potable (drinking) water according to the EU Council Directive of 1998, Rajiv Gandhi National Drinking Water Mission (India) and World Health Organisation Guidelines for Drinking Water (Table 1.1) is defined as water having a total absence of faecal indicator bacteria in 100 mL of sample after disinfection using chemical or/and physical methods (Vasudevan and Pathak, 1998; van Buren *et al.*, 2005).

Table 1.1 Guidelines for the microbiological quality of drinking water*

Microorganism	Permitted limit	Permitted value	Comments
A. Piped water supplies			
A1 Treated water entering the distribution system			
Faecal coliforms	Number per 100 mL	0	Turbidity 1 NTU: for disinfection with chlorine pH preferably 8.0: free chlorine residual 0.2-0.5 mg/litre following 30 minutes contact.
A2 Untreated water entering the distribution system			
Faecal coliforms	Number per 100 mL	0	In 98% of samples examined throughout the year in the case of large supplies when sufficient samples are examined.
Coliform organisms	Number per 100 mL	0	
Coliform organisms	Number per 100 mL	3	In an occasional sample, but not in consecutive samples.
A3 Water in the distribution system			
Faecal coliforms	Number per 100 mL	0	In 95% samples examined throughout the year in the case of large supplies when sufficient samples are examined.
Coliform organisms	Number per 100 mL	0	
Coliform organisms	Number per 100 mL	3	In an occasional sample, but not in consecutive samples
B. Unpiped water supplies			
Faecal coliforms	Number per 100 mL	0	Should not occur repeatedly if occurrence is frequent and if sanitary protection cannot be improved an alternative source must be found if possible.
Coliform organisms	Number per 100 mL	0	
C. Bottled drinking water			
Faecal coliforms	Number per 100 mL	0	Source should be free from faecal contamination
Coliform organisms	Number per 100 mL	0	
D. Emergency water supplies			
Faecal coliforms failure	Number per 100 mL	0	Advise public to boil water to meet guideline values, if necessary
Coliform organisms	Number per 100 mL	0	
Enteric viruses	Number per 100 mL	0	

* WHO guidelines, modified from Vasudevan and Pathak, 1998

1.6 Risk Assessment for pathogenic micro-organisms

Risk assessment is defined as the chance that a substance or a microorganism will adversely produce harm or disease under specified conditions (Percival *et al.,* 2004). Microbial risk assessment was developed from a simple model based upon chemical risk assessment and consists of four basic steps which are:

(1) ***Hazard identification***: this approach identifies the likely micro-organism(s) of concern or agents of potential significance, using scientific literature including: clinical studies; epidemiological studies; surveillance, etc. The outcomes of hazard identification may then be used for quantification of risk assessment for example: infection without apparent illness; morbidity or mortality in the susceptible general population or sub- populations. Both infection and mortality in the general population from these infectious agents may be considerable (Hass *et al.,* 1993; 1999) and measures should be taken to overcome the particular hazards identification (WHO, 2004c).

(2) ***Dose response***: this describes the time period and severity of harmful effects that would be the outcome if the micro-organism were ingested from water, using quantitative or qualitative methods. Furthermore, the relationship between the microbe and human host is characterised using factors such as virulence and infectivity. Thus low doses of some micro-organisms may lead to severe infection, for example typhoid due to *Salmonella typhi* (Ivnitski *et al.*, 1999).

(3) ***Exposure assessment***: This approach determines the extent of human exposure i.e. the likely microbial doses consumed by hosts as a result of drinking contaminated water. The levels of micro-organism and the actual consumption rate of drinking water is assessed.

(4) ***Risk characterization***: This step combines the information obtained from exposure assessment and dose response into an overall estimation of the likely adverse effect. This can be done by calculating the number of organisms ingested and then combining it with the dose response parameter.

The results of risk characterization can then be used for risk management e.g. a *Cryptosporidium* case study was organized in New York city, US, using all of the above parameters (Percival *et al.*, 2004; WHO, 2004c). Thus the above microbial risk assessment model can be applied to practical risk management to:

- predict endemic rates of water-borne infections;
- calculate micro-organism dose for standard treatment protocols;
- ascertain the effectiveness of treatment of water;
- forecast the likely risk if there is a failure in water treatment.

1.7 Traditional culture-based methods for the detection of faecal indicator bacteria from water

Established methods for the detection of the coliform group are based on the fermentation of lactose with gas and acid production at 35°C or 37°C (Clesceri *et al.*, 1998; Anon., 2002). Two traditional, routinely used standard methods for the enumeration and detection of coliforms from water samples using these characteristics are multiple tube fermentation, or most probable number (MPN), and membrane filtration (MF).

1.7.1 Multiple tube fermentation or most probable number technique (MPN)

The US Public Health Service Drinking Water Standard in 1914 introduced this bacteriological method which specified that not more than one out of five 10 ml portions of any sample examined should show the presence of *B. coli* (now *E. coli*) by the specified most probable number procedure (WHO, 2004a). In this method, measured volumes of the sample at one or more dilutions are inoculated into a series of bottles or tubes containing a suitable differential liquid medium. This method is based on two or three steps namely, (i) presumptive, (ii) confirmed and (iii) completed tests.

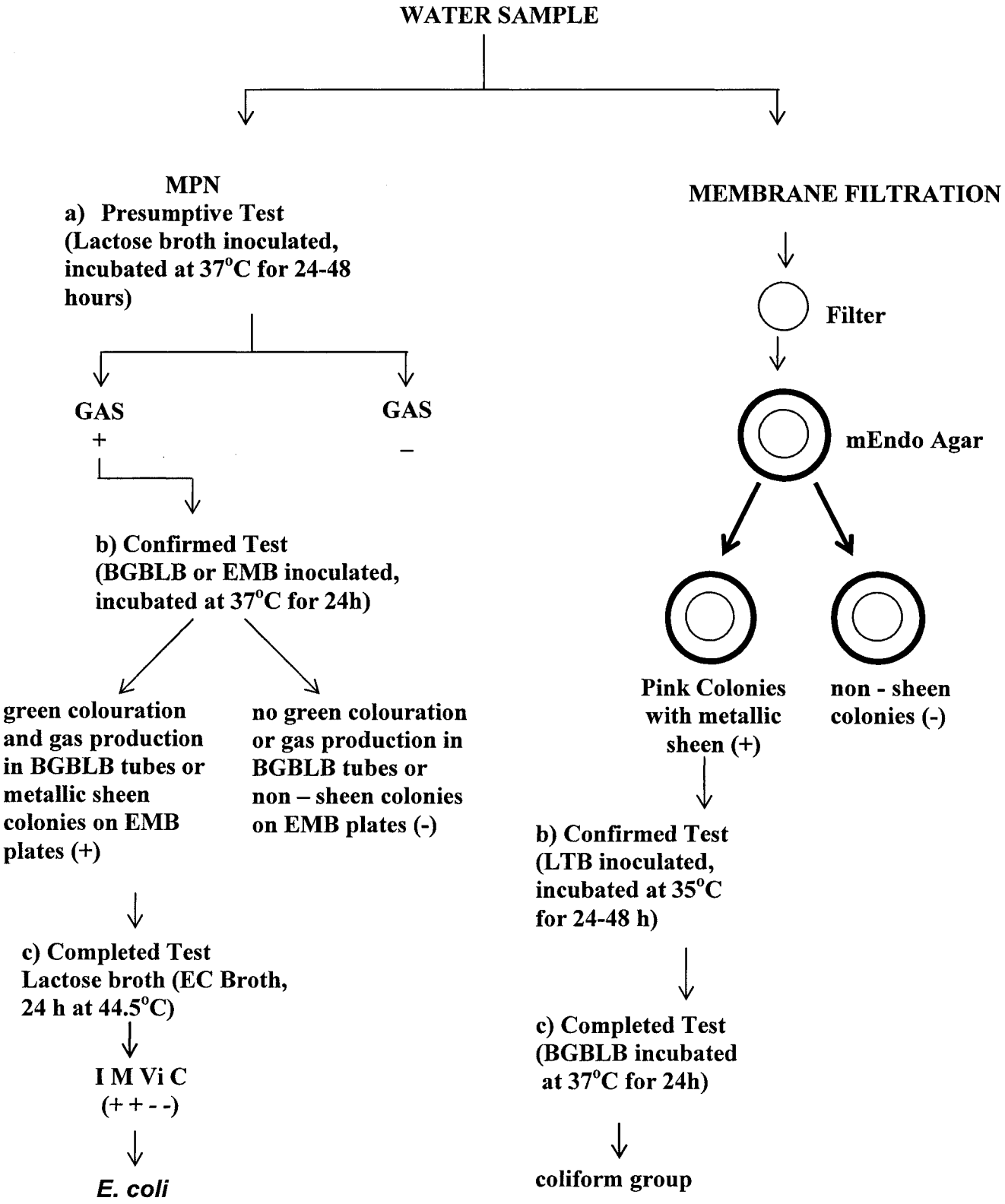
In the presumptive step, tubes are filled with a lactose-based medium with a pH indicator (acid) and inverted Durham tubes as an indicator for gas production. The lactose medium used in the US is lauryl tryptose lactose broth, LTLB (Clesceri *et al.*, 1998). The equivalent UK method uses minerals modified glutamate broth (Anon., 2002) whereas in India MacConkey broth is generally used (Vasudevan and Pathak, 1998). The use of MacConkey broth is not recommended in the UK and US as it contains peptone and bile salts that have inhibitory properties, with significant batch variation (Chilvers, 2001; Anon., 2002). The tubes or bottles are then incubated at 37°C for 24-48 hours. A set of tubes in the above presumptive test with gas production and colour change due to acid production are recorded as positive and are then used to obtain statistical presumptive data using a most probable number table as formulated by McCrady in 1915 (Clesceri *et al.*, 1998). The results usually are expressed as the most probable number (MPN) of cells per 100 mL.

The confirmatory step uses positive tubes from the above step, which are sub cultured in tubes of confirmatory medium. In India brilliant green bile lactose broth

(BGBLB) or eosin methylene blue agar (EMB) are used, with incubation at 37°C for 24-48 hours in the confirmatory step. After the incubation period green colouration and gas production in the case of BGBLB tubes or colonies with a green metallic sheen on EMB agar plates indicates a positive result (Vasudevan and Pathak, 1998). In the UK, ortho-nitrophenyl-β-galactopyranoside (ONPG) broth is used with incubation at 37°C for 24 hours, whereas lauryl tryptose lactose broth (LTLB) at 35°C for 24 hours is used in the US for confirmation of total coliforms (Anon., 2002; Clesceri *et al.*, 1998). A number of techniques can be used to differentiate faecal coliforms (FC) from total coliforms (TC), such as the Eijkman test which uses lactose broth incubated at 37°C and 44°C.

In India, the completed test is performed using colonies transferred to EC broth for the completed test for *E. coli* as shown in the flow chart in Figure 1.3. The EC broth tubes are incubated for 24 hours at 44.5°C. Positive tubes showing turbidity and gas production confirm the presence of *E. coli*. Additionally, biochemical tests such as the IMViC (indole, methyl red, Voges - Proskauer and citrate) tests can be used for the differentiation of other faecal coliforms (Vasudevan and Pathak, 1998; WHO 2004b). A combination of all positive tubes from lactose broth and EC broth tests will be noted and compared using MPN tables, to give the completed count of *E. coli*.

Figure 1.3 Flow chart for detection of coliforms by traditional culture-based methods used in India



BGBLB - brilliant green bile lactose broth
EMB – eosin methylene blue agar
LTB - lauryl tryptose lactose broth

Detection of enterococci (faecal streptococci) by the MPN method uses azide dextrose broth for the presumptive step with incubation at 35 $\pm$0.5°C for 24-48 hours. Pfizer selective enterococcus (PSE) agar is used for the confirmatory step in the US and India (Vasudevan and Pathak, 1998; Clesceri *et al.*, 1998). In contrast, the UK standard methods perform only membrane filtration for detection and enumeration of enterococci, (Anon., 2002) as described on page 1-23.

Clark in 1967 introduced a simple modification of the most probable number method called the presence- absence (P-A) test. For the presumptive phase the test uses double-strength MacConkey broth, modified by the addition of tryptone at 10 g L^{-1}. This P-A test uses dilution bottles containing an inverted gas collector tube (a Durham tube) and 50 mL of double-strength modified MacConkey broth to which 50 mL of water sample is added. Each bottle is incubated at 35°C for five days and checked each day for acid and gas production. For the confirmatory phase all positive bottles are inoculated into confirmatory broths such as BGBLB, EC broth, MacConkey broth and then streaked onto MacConkey agar. All confirmatory broths are incubated at 35°C for 48 hours and agar at 35°C for 24 hours.

The MPN method, although it uses five tubes, is not a fully quantitative test as it gives a statistical estimate of the most probable number of bacteria present in a sample, rather than a formal count. It is quite tedious and labour intensive, as many dilutions and steps have to be used. Interference by non coliform bacteria (Evans *et al.,* 1981) coupled with the inhibitory nature of the media used (McFeters *et al.,* 1982) can contribute to underestimation of coliform bacteria. Furthermore, the outcome of the result is achieved only after 72 hours, making the test extremely slow in operation.

1.7.2 Membrane filtration (MF) method

This technique was introduced after 1950 and since then has been accepted and approved for monitoring the microbial quality of drinking water in many countries due to its ease of use, compared to MPN-based methods. A measured volume of water sample, usually 10-100 mL, is filtered through a sterile membrane of pore size 0.45 μm which is then transferred to the surface of a solid medium or to an absorptive pad of broth medium in a Petri dish. Then the above Petri dish with membrane filter and growth medium is incubated at 35 ± 0.5°C for 20-24 hours for total coliforms (Clesceri *et al.*, 1998). Confirmed and completed steps can be carried out as mentioned in the MPN method above.

In the UK (Anon, 2002), in the presumptive phase membrane lauryl sulphate broth (mLSB) is used which is a rich medium containing lactose (3% w/v), phenol red as a pH indicator, turning yellow when acid is produced from lactose, and sodium lauryl sulphate (0.1% w/v) for the inhibition of Gram-positive bacteria. The US standard method uses m-Endo agar for the detection of total coliforms which is also a highly rich lactose medium containing basic fuchsin as the pH indicator, sodium lauryl sulphate and sodium deoxycholate, both of which are used for inhibition of Gram-positive bacteria (Chilvers, 2001). For the detection of faecal coliforms and *E. coli* the use of m-FC medium is recommended, along with an elevated temperature i.e. 44.5°C for 24 hours (Rompre *et al.*, 2002).

In India, m-Endo broth saturated in absorbent pads is used in the presumptive phase, which is incubated at 35 ± 0.5°C for 20-24 hours as shown in Figure 1.3 (Vasudevan and Pathak, 1998). Typical pink to dark red colonies with a metallic sheen from the Petri plates in the presumptive phase are transferred to LTB in the confirmatory

phase with an incubation period of 24-48 hours at 35°C. Positive tubes from the confirmatory phase are transferred to BGBLB broth in the completed phase. BGBLB broth tubes with gas production will verify the presence of total coliforms.

The following formula is used to determine the density of bacteria in the water sample:

$$\text{Total coliform colonies/100 mL} = \frac{\text{Coliform colonies counted x 100}}{\text{Volume of sample filtered (mL)}}$$

The US standard method uses mE agar or mEnterococcus agar for isolation of enterococci in the presumptive step with incubation at 41± 0.5°C for 48 h (Clesceri *et al.*, 1998). Confirmation or verification of enterococci is carried out using brain heart infusion broth/agar, aesculin hydrolysis and biochemical testing. In the UK, mEnterococcus agar (at 44°C for 48 h) is used in the presumptive step. Bile aesculin agar or kanamycin aesculin azide agar at an incubation temperature of 44°C for 18 h, producing dark brown or black haloes around colonies indicating the hydrolysis of aesculin is used in the confirmatory step for enterococci. Additional differentiation tests such as 40% bile tolerance, or growth at pH of 9.6, or 6.5% of sodium chloride tolerance and biochemical tests are performed in the completed step (Anon, 2002).

The membrane filter (MF) technique is highly reproducible, can be used to test relatively large volumes of water samples, and usually yields qualitative or quantitative (numerical) results more rapidly than the multiple-tube fermentation procedure. It has also been adapted for field use, e.g. using a portable incubator such as the DelAqua field kit commercially available from Oxfam (Wegelin *et al.*, 2000; Godfrey, 2003). The MF technique is extremely useful in monitoring drinking water and a variety of natural waters with the exception of highly turbid waters, where the filter may become blocked. The results are often obtained within 24 hours as opposed to 48-72 hours for MPN. Some negative points of the MF technique have

been noticed including: a decrease in coliform recovery because of the presence of high number of background heterotrophic bacteria (Clark, 1980); and excessive crowding of colonies on m-Endo causing a decrease in the number of coliform colonies producing a metallic sheen (Burlingame *et al.,* 1984).

1.7.3 H_2S Strip test (simple field test method)

Manja *et al.* (1982) proposed a simple, rapid, ready-to-use, inexpensive field test for the detection of organisms producing hydrogen sulphide such as *Salmonella, Proteus, Citrobacter* and some strains of *Klebsiella* from drinking water (Pillai *et al.*, 1999; Nair *et al.*, 2001). They observed that faecal coliforms in drinking water are consistently associated with organisms that produce H_2S. Thus on the basis of such observations they formulated a single bottle assay as a simple presence-absence test (Rijal *et al.* 2000; Manja *et al.*, 2001). The medium has been recommended by World Health Organisation (2002a) and Indian organizations such as the Rajiv Gandhi National Drinking Water Mission (Vasudevan and Pathak, 1998) for detection of faecal indicator bacteria in drinking water samples.

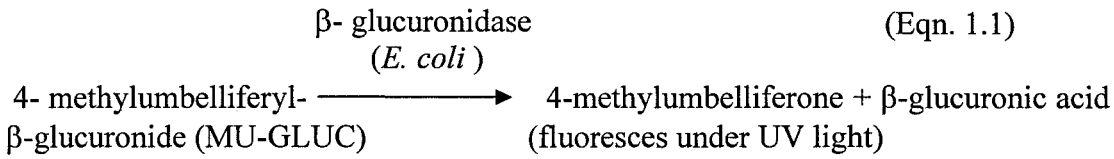
1.8 Modern methods for the detection of faecal indicator bacteria

While culture-based methods have been used since the early days of microbiology in the nineteenth century, more recent developments have given rise to a number of alternative techniques by which faecal indicator bacteria may be investigated, based on the detection of enzyme activity, or the presence of certain bio-molecules in a sample.

1.8.1 Defined enzyme substrate technology based on culture medium

This method was introduced by Edberg *et al.*, (1988, 1990) and is a technique whereby the presence of a micro-organism possessing a specific enzyme can be detected by using a particular substrate e.g. the enzyme β-galactosidase present in

coliforms can be detected by addition of a suitable β-galactoside substrate. The incorporation of such substrates into a selective medium used for detection and enumeration can eliminate the need for subculture and further biochemical tests (Manafi, 1996, 2000) and thus the results may be obtained in a single step. Two types of substrates commonly used are (i) fluorogenic and (ii) chromogenic enzyme substrates. The fluorogenic substrate such as 4-methylumbelliferyl-β-galactoside (MU-GAL) consists of a galactose sugar molecule coupled to a fluorogen which is able to convert UV light to visible light once it has been cleaved by a specific enzyme, in this case β-galactosidase (Alonso *et al* 1999; Manafi *et al.*, 2000). Methlyumbelliferone substrates are water soluble, highly sensitive and very specific. These are generally preferred for use in liquid media as they diffuse rapidly in agar media (Manafi, 1996). Another example is given below for the enzyme β-glucuronidase.



(Eqn. 1.1)

Chromogenic enzyme substrates are compounds which are specific substrates for particular enzymes and which change colour due to the action of a chromogen, which produces its colour when cleaved by an enzyme in a manner equivalent to that shown above for the fluorogenic methylumbelliferone (MU) substrates. The chromogenic substrates are often indolyl derivatives such as 5-bromo-4 chloro-3-indolyl-β-glucuronide (X-GLUC), 5-bromo-6-chloro-3-indolyl-β-glucuronide (magenta-GLUC) and 6-chloro-3-indolyl-β-glucuronide (salmon-GLUC) commonly used for enumeration of *E. coli* and indolyl-β-D-glucoside used for the enumeration of *Enterococcus faecalis* (Ley *et al* 1993; Rhodes, 1997). Total coliform detection can be achieved by the chromogenic detection of β-galactosidase which catalyses the

breakdown of lactose to galactose and glucose e.g. coliforms such as *E. coli* can degrade the chromogenic substrate *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) via the enzyme β-galactosidase into a yellow-coloured compound *ortho*-nitrophenol and galactose.

(Eqn. 1.2)

ortho-nitrophenyl-β- galactopyranoside (ONPG) —(β-galactosidase (coliforms))→ orthonitrophenol + galactose (yellow colouration)

Thus *E. coli*, which possesses both enzymes i.e.β-galactosidase and β-glucuronidase can be distinguished from other coliforms as they possess only β-galactosidase. Faecal enterococci contain the enzyme β-glucosidase that hydrolyses e.g. 4-methylumbelliferyl-β-D-glucoside (MU-GLU) and releases 4- methylumbelliferone which exhibits fluorescence under UV. Aesculin (6, 7-dihydroxycoumarin-6-β-glucoside) can also be hydrolysed by enterococci (Facklam and Elliot, 1995) to release aesculetin (6, 7-dihydroxycoumarin) which reacts with Fe^{3+} ions to form dark brown- or black-coloured complexes (Hartman *et al.,* 1992). James *et al.* (1996) used 3,4-cyclohexenoesculetin-7-β-D-glucoside incorporated into solid media using the above principle, which formed a non-diffusible end product in the presence of iron. Similarly Dufor (1980) incorporated indoxyl-β-D-glucoside a chromogenic substrate into mEI agar. Many commercial kits such as Enterolert (IDEXX Laboratories, Westbrook, ME, USA), Quanti Tray (IDEXX) and microtitre plate MUST (Sanofi, Paris, France) use the fluorogenic indicator substrate MU-GLU for the detection of β-D-glucosidase from enterococci (Manafi, 2000).

1.8.2 Direct determination of enzymatic activity using fluorimetry

George *et al.* (2000) used fluorogenic substrates such as MU-GAL and MU-GLUC for direct enzymatic detection of faecal coliforms in freshwaters. The method used an automated analyzer, Colifast CA-100 (Colifast systems, Oslo, Norway), for estimation of the level of microbial contamination of water by coliforms. Here, the sample is filtered and incubated at 37°C for total coliforms (TC) and 44°C for faecal coliforms (FC) in a growth medium selective for coliforms and containing MU-GAL. Fluorescence due to MU-GAL hydrolysis depends upon number of coliforms in the sample, taking 2 hours for samples with high numbers of coliforms and 11 hours for 1 culturable total coliform in a sample (Rompre *et al.*, 2002).

1.8.3 Detection of coliforms by enzymatic methods using solid-phase cytometry

Van Poucke and Nelis (1999) developed a method using a laser-scanning device (ScanRDIT-Chemunex, Ivry-sur-Seine, Paris, France) which detected and enumerated low numbers of fluorescently labeled cells by using a solid phase cytometry technique to decrease the time required for analysis of an enzymatic membrane filtration test. This system allows very low quantitative detection of fluorescently labeled cells between 1- 1000 per sample, and even cells in a non-culturable state. Thus *E. coli* cells are filtered and trapped onto 0.4 μm pore size filter, treated for 3 hours at 37°C with reagents to induce activity of β-glucuronidase and detected using a fluorogenic enzyme substrate.

1.8.4 Molecular methods

These methods increase the rapidity of analysis and are highly sensitive and specific without using cultivation or confirmation steps. These are:

(a) ***Immunological methods***: using specific recognition between antibodies and antigens, in one of two ways:

- by performing immunocapture of cells or antigens by enzyme-linked immunosorbent assay (ELISA), or by detecting target cells by immunofluorescence assay (IFA) or immuno-enzyme assay (IEA).
- Immunomagnetic separation (IMS), to remove and concentrate cells from a water sample prior to detection.

The ELISA method for coliforms, as developed by Obst *et al.* (1989), uses a monoclonal antibody against the enterobacterial common antigen (ECA), a lipopolysaccharide found in the outer membrane of the Enterobacteriaceae. The limitations of this method are that the solid matrix leads to non-specific binding for the second antibody and the assay depends upon the specificity of antibody used, the concentration of antigen and antibody and the nature of the reaction solution.

Immunofluorescence methods allow detection and enumeration of a single specific cell using a direct or indirect procedure (Suzuki *et al.*, 2005). A specific antibody is directly conjugated with a fluorochrome in the direct method, while the indirect procedure binds a specific primary antibody to the target antigen followed by the addition of a fluorochrome-labeled secondary antibody against the first antibody. Secondary antibodies can be obtained

from commercial suppliers with a range of bound fluorochromes (Rompre *et al.*, 2002).

The IMS method takes a different approach for rapid identification of culturable and non-culturable micro-organisms (Safarik *et al.*, 1995). The principle of this method is based on using magnetic beads coated with monoclonal or polyclonal antibodies. Purified antigens are biotinylated and then bound to strepavidin- coated paramagnetic particles, for example Dynal™ beads. The sample to be tested is gently mixed with the immunomagnetic particles or beads. Target organisms are recovered using a magnet whereas the non-bound materials are left behind. This method has been used for the recovery of *E. coli* O157 from drinking water (Anon., 1996). Pyle *et al.* (1999) suggested a combination of IMS and solid-phase laser cytometry for the detection of *E. coli* O157 in water.

(b) ***Nucleic acid based methods*:** These methods use molecular hybridization between complementary sequence of a nucleic acid probe and a nucleic acid target e.g. a chromosomal DNA sequence found in a particular bacterium. The most frequently used nucleic-acid-based methods are:

Polymerase chain reaction (PCR): This method employs enzyme-catalyzed *in vitro* DNA synthesis to make millions of identical copies of DNA. By knowing the base sequence of adjacent regions of DNA it is possible to construct synthetic oligonucleotide primers complementary to the flanking regions of a target sequence (Reed *et al.*, 2003). The target DNA fragment is amplified by a cycling replication which consists of reactions catalyzed by a

thermostable DNA polymerase (e.g. *Taq* polymerase), using oligonucleotidic primers. Specificity depends upon the hybridization temperature and the degree of complementarily between the target and the primers. Amplification is performed using nucleic acids generated by cellular lysis followed by chemical extraction. PCR amplification steps involve (i) DNA denaturation from double to single strand, by heat-treatment, (ii) annealing of primers to the single-stranded DNA at a specific hybridization temperature, and (iii) extension of primer by DNA polymerase. The whole amplification process requires 20 to 40 temperature cycles of amplification. PCR products are identified using agarose gel electrophoresis. Primers based on the *lacZ* gene can be used for detection of coliforms and the *uidA* gene for detection of *E. coli* (Bej *et al.,* 1991; Rompre *et al.*, 2002).

In situ hybridization techniques (ISH): this method uses oligonucleotide probes for the detection of complementary nucleic acid sequences by annealing to one another to form hybrids. These oligonucleotide probes are selected to be specific and complementary nucleic acids unique to a micro-organism, species or group. Researchers rely on computer-aided sequence comparisons, available in various data gene bank (Rompre *et al.,* 2002). Presently rRNA *in situ* hybridization uses fluorescent-labeled nucleotide probes to detect hybridization (FISH). Advantages of FISH are its sensitivity, speed of visualization of single cells, stability of hybridization products, safety, availability of multiple labels and ease of use (Richardson *et al.,* 1991; Swinger and Tucker, 1996; Roslev *et al.*, 2004). The FISH procedure uses cell fixation, hybridization, post-hybridisation washing and detection. rRNA target probes such as the EC1531 probe (Poulsen *et al.,* 1994) are used for the

detection of *E. coli*; this probe is complementary to a 23S rRNA sequence in this bacterium, composed of 20 nucleotides with a C+G content of 55%.

1.9 Detecting damaged and injured indicator bacteria in the environment

Faecal indicator bacteria may become injured in environments that are nutritionally limiting and cannot support their growth, such as in natural waters, including drinking water sources (LeChevallier *et al.*, 1983), surface waters (Bissonnette *et al.*, 1975), and after exposure to a variety of stressful factors, such as chlorine disinfectants (Calabrese and Bissonnette, 1990); heat (Czechowicz *et al.*, 1996; George and Peck, 1998), sunlight (Fujioka *et al.,* 1981; Fujioka and Narikawa, 1982; Reed and Meyer 2000), heavy metals (MacLeod *et al.,* 1967; Calabrese and Bissonnette, 1990b), and starvation (Bloomfield *et al.,* 1998) These injured bacteria may become more sensitive to the selective ingredients present in routinely used enumeration media (McFeters *et al.*, 1986) e.g. pararosanline and sodium sulphite present in m-Endo were noted to be inhibitory for growth of *E. coli* NCTC8912 (Khaengraeng, 2004). Some other researchers have commented that recovery of injured cells of *E. coli* O157 can be inhibited by selective agents present in enumeration media, which may be overcome by the incorporation of a non-selective pre-enrichment step (Blackburn and McCarthy, 2000).

Resuscitation methods have been used by different researchers for culture-based enumeration of injured bacteria e.g. the growth of such bacteria may be enhanced by initially culturing the organism in an enriched, non-inhibitory medium at moderate temperature followed by the use of a selective medium and/or temperature (Clesceri *et al.*, 1998). Resuscitation of heat-injured bacteria has also been achieved using a

non-selective agar overlay method (Rose *et al.*, 1975) or non-selective agar underlay (Kang and Siragusa, 1999). LeChevallier *et al.* (1983) formulated a new medium named m-T7 for enhanced recovery of injured coliform bacteria from drinking water, based on the less inhibitory selective agent tergitol-7 instead of bile or other surfactants. The Standard Methods for Examination of Water and Wastewater in the US (Clesceri *et al.*, 1998) contains a section on recovery enhancement of injured bacteria where different methods are discussed e.g. for *E. coli* and faecal streptococci, temperature acclimation in two-layer agar using a non-selective overlay medium without glucose i.e. (tryptic soy agar or nutrient agar) over a selective agar (mFC, m-*Enterococcus* agar) with a 2 hour incubation at 35°C followed by 22 hours incubation at 44.5°C. In addition, the elimination of rosolic acid (acting as a suppressive agent) from original mFC agar, is recommended for the enumeration of injured bacteria. Furthermore, temperature acclimation for the improved recovery of injured bacteria using the less-inhibitory medium m-T7 agar with 8-hour incubation at 37°C followed by 12-hour incubation at 44.5°C. A lower initial temperature incubation period of 4 hour at 30°C followed by 14 hour at 37°C is also recommended in the UK (Anon, 2002).

In addition to the inhibitory effects of selective media on injured bacteria discussed above, the decrease in the count of damaged bacteria on plates of agar-based media enumerated under standard aerobic conditions may also be explained in part by taking into account that injured bacteria exposed to variable stresses may enter a state on transfer to nutrient-rich medium, where they undergo respiration-induced death because of the production of a burst of intracellular reactive oxygen species (ROS), derived from respiratory activity, which is uncoupled from growth (Bloomfield *et al.*, 1998; Aldsworth *et al.*, 1999). Culturing these injured bacteria under anoxic

conditions in presence of ROS-scavengers provides an alternative way for such microbes to grow under ROS-neutralised conditions (e.g. Czechowicz *et al.*, 1996; Mizunoe *et al.*, 1999; Stephens *et al.*, 2000; Khaengraeng, 2004; Khaengraeng and Reed, 2005). This aspect is considered in greater detail in Chapters 3 and 4.

The multiple stresses frequently present in the environment expose bacteria to different types of extreme conditions and only those bacteria that can endure these stresses can survive and thus remain viable. Conventionally, viability is assessed by culturing the organism under laboratory conditions (Section 1.7), to produce a visible result (e.g. a colony on an agar plate, or a turbid broth). However, there is some controversy as to whether this is the most appropriate means of assessing viability. Death, a irreversible state, is a term applied to cells which become moribund or which lose their culturability but a state between life and death may exist for those organisms which are not culturable at a given time or under prevailing stressful conditions and which may revert to a culturable state when supplied with appropriate conditions, for example resuscitation. This has been called the viable but non-culturable state or VBNC (Oliver, 1995; Oliver and Bockian, 1995; Aertsen and Michielis, 2004). The VBNC as defined by Oliver (1993) is: "a state where a bacterial cell is metabolically active but is incapable of undergoing sustained cellular division required for growth in or on a medium normally supporting growth of that cell". Xu *et al.* (1982) were among the first researchers to bring to light the possible existence of the VBNC state through experimental evidence. Since then the biological meaning of the viable but non-culturable state has been a source of considerable debate about the physiological significance and origin of this state (Bogosian and Bourneuf, 2001). Workers such as Roszak and Colwell (1987) proposed the idea that VBNC bacteria could represent an important sub-population

of cells which cannot grow on routinely used growth media and which cannot be enumerated by traditional resuscitation methods. The bacteria in VBNC state still may regain their biological activity e.g. cell elongation, and which can only be detected using other methods, e.g. based on their metabolic activity. As an example, a difference between protein expression patterns of stationary phase and VBNC cells was noted in *Enterococcus faecalis*, thus representing both to be physiologically distinct states (Heim *et al.*, 2002). In contrast, the opponents of this hypothesis believe VBNC to be "a stable state of injury" limited to a short duration and ultimately leading to lethal injury and death. As an example, studies performed using starved *E. coli* and *Salmonella typhimurium* cells indicated a progressive deterioration rather than a distinct VBNC state (Nystrom, 2001).

The factors that have been reported as inducing bacteria to enter the VBNC state are: low temperatures in stationary phase *Vibro vulnificus* (Wolf and Oliver, 1992; Oliver, 2005); moderate temperatures of 37°C in *Campylobacter jejuni* in aerobic stream water (Rollins and Colwell, 1986); physiological age of the culture and external salt levels in enteric bacteria maintained in natural seawater microcosms (Xu *et al.*, 1982); and low nutrient levels and/or natural light in *E. coli* (Arana *et al.*, 1992; 1999). Several methods have been introduced for determination of the VBNC state of bacteria e.g. the direct viable count (DVC) method (Kogure *et al.*, 1979; Lleo *et al.*, 2005) where, in the presence of suitable concentrations of nutrients and nalidixic acid, viable bacteria continue to grow without cell division and thus form elongated cells which are then counted by epifluorescence microscopy. Singh *et al.* (1990) have reported a similar approach using yeast extract-nalidixic acid for the enumeration of chlorine-injured bacteria. A further method is based on the use of fluorescent probes for direct detection of actively respiring bacteria using redox dyes

such as 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) which acts as an artificial electron acceptor to directly compete with molecular oxygen (Besnard *et al.*, 2000). Another method combines the DVC method with the use of monoclonal antibodies linked to a fluorescent compound (Xu *et al.,* 1982; Roszak *et al.,* 1984) while a further approach is the acridine orange direct count (AODC), where morphological integrity of bacterial cell is confirmed (Gonazales *et al.,* 1992; McDougald *et al.,* 1998).

A major aspect of the VBNC hypothesis is the question as to whether such cells are able to resuscitate and grow under certain conditions and therefore whether they might represent a potential source of pathogenic microbes (e.g. in the case of water-related diseases). Resuscitation of non-culturable cells as defined by Bogosian *et al.* (2000) is a major aspect of the VBNC hypothesis and is defined as the conversion of non-culturable cells into culturable cells without any change in cell numbers resulting from re-growth. It has been suggested that the presence of culturable cells is necessary for the resuscitation of non-culturable cells, possibly due to a factor that is produced by culturable cells which triggers the recovery of non-culturable cells. Some researchers disagree with the suggestion that bacterial cells in the VBNC state can regain their pathogenicity or resuscitate from that state by explaining that recovery is likely to be due to the re-growth of a few viable cells in the original cell cultures (Morgan *et al.,* 1991; Weichart *et al.,* 1992; Sylvester *et al.*, 2001). Others such as McDougald *et al.* (1998) suggest the existence of two phases within the VBNC response: firstly, the transition of viable cells to "young VBNC" cells, with loss in culturability but with the retention of cellular integrity and nucleic acids as well as their ability to cause disease (e.g. pathogenicity was retained in VBNC *E. coli* after introduction into ligated rabbit ileal loops; Grimes and Colwell, 1986) and

secondly, a "latent VBNC" state where debilitation of cellular integrity, DNA and RNA occurs, leading to loss of viability. Thus as the cells persist longer in the VBNC state they may lose both their culturability and their potential for infectivity.

In one of the best studied examples, it has been suggested that non-culturable cells of *Vibrio vulnificus* formed at low temperatures and low nutrient levels could be resuscitated with a temperature up shift (e.g. Whitesides and Oliver, 1997; Oliver, 2005). However, this has been questioned by Bogosian and Bourneuf (2000) who commented that warming or temperature up shift permitted injured cells to grow on nutrients provided by dead cells, thus giving an illusion of resuscitation of non-culturable cells. More recently, Kong *et al.* (2004) have suggested that the resuscitation of *V. vulnificus* from the non-culturable state may be due to the production of starvation-induced stress proteins. Roszark *et al.* (1984) reported the recovery of non-culturable cells of *Salmonella enteritidis* by the addition of nutrients e.g. adding heart infusion broth to sub-samples of river water. Bogosian *et al.* (2000) noticed a substantial increase in the peroxide-sensitive cell count of *V. vulnificus* cultures when scavengers of peroxides such as catalase or sodium pyruvate were incorporated into the enumeration media. Mizunoe *et al.* (1999) reported a similar effect with non-culturable, starvation-stressed and low-temperature-stressed *E. coli* O157 cells, which regained culturability after treatment with H_2O_2-degrading agents or antioxidants. Exogenous addition of ROS-quenching compounds may provide protection against respiratory oxidative stress (Aldsworth *et al.*, 1999) and thus may improve the recovery of the "non-culturable" cells. All of the above points support the notion that VBNC cells may resuscitate under certain conditions and thus acquire potential pathogenicity as indicated in *in vivo* experiments where virulence is retained (e.g. McDougald *et al.*, 1998). However, such observations also place a

question mark over the whole concept of the VBNC state. Thus Bogosian *et al.* (1998) explains that viable but non-culturable cells may simply be non-culturable under certain specific growth conditions, e.g. using routinely used growth media. Subsequently, Oliver (2000) suggested that non-culturability *of V. vulnificus* may result from placing cells in/on high nutrient media, due to the production of reactive oxygen species, and Kong *et al.* (2004) have subsequently demonstrated the central role of catalase in resuscitation from the non-culturable state in this organism, suggesting that the definition of VBNC cells may depend upon the culture conditions used, and also identifying a significant role for oxygen-dependent phenomena in defining the VBNC state.

1.10 Aims of the study

The overall purpose of this project was to investigate whether the methodology for detecting and enumerating faecal indicator bacteria in water samples in India could be improved, and to provide information on the validity of current methods alongside alternative/novel methods that might give enhanced counts for injured bacteria. A major aspect to consider was the effect of conventional aerobic culture on the growth of injured bacteria and the possible enhancement under ROS-neutralised conditions (Dodd *et al.*, 1997).

The specific aims of the present research were:

1. To study the inactivation of faecal indicator bacteria in traditional Indian water storage vessels of different composition (e.g. brass, earthern), with enumeration of injured bacteria in different media under standard aerobic conditions and under conditions designed to neutralise reactive oxygen species (ROS).
2. To investigate the effects of various environmental factors, e.g. sunlight, temperature, chlorine disinfection, pH, starvation, on the counts of faecal

indicator bacteria, using non-selective and selective media in aerobic conditions and under ROS-neutralised conditions.

3. To test currently available methods for the resuscitation of injured faecal indicator bacteria, in comparison with standard US and UK methods and with methods based on ROS-neutralisation.
4. To use the information gained from the investigations outlined above to develop a novel broth-based assay for faecal coliforms and/or *E. coli* and then to compare this in an MPN multiwell format against an appropriate agar-based medium under laboratory conditions.
5. To evaluate the use of such a medium in a field-based broth-type format, in comparison to the currently available field test method, (H_2S broth) and alongside a conventional plating method, including evaluation of the operation of the field-based medium with unskilled personnel in rural locations in India as a preliminary field trial of this approach.

Chapter 2

General Materials and Methods

This chapter outlines and describes common materials and methods used in the majority of experiments in the present study. The precise materials and methods related only to specific experiments are described in the appropriate Chapter.

2.1 Bacterial cultures and preparation of cell suspensions

2.1.1 Bacterial cultures and their maintenance

The principal micro-organism used in the current study was *Escherichia coli* strain NCTC8912. This strain was originally obtained from the National Collection of Type Cultures, Colindale, UK and studied at the Northumbria University, UK. The other strains of *E. coli,* used for comparison, were strain TN675 obtained from the Central Research Division, Osaka, Japan and two wild isolates PUCC061 and PUCC113, which were isolated from local natural waters in Panjab and serotyped by the National *Salmonella* and *Escherichia* typing centre at the Central Research Institute, Kasauli, Himachel Pradesh, India. The last three were studied at Panjab University, Chandigarh, India and were maintained in the Panjab University Culture Collection (PUCC).

The second micro-organism studied was *Enterococcus faecalis* NCTC775, a laboratory strain originally obtained from the National Collection of Type Cultures, Colindale, UK and studied at Northumbria University. The other three strains of *E. faecalis* used were strain ATCC 35550, which was obtained from IMTECH (Indian Institute of Microbial Technology), Panjab whereas the wild isolates PTO1 and PTO2 were isolated from natural waters. Thus a total of two bacterial species and four strains of each species, consisting of two standard strains and two wild isolates, were used in the present study. Stock cultures of all of these strains were maintained by repeated subculture on plates of nutrient agar (Oxoid, Basingstoke, UK or

Labchem Himedia, Mumbai, India), using the streak dilution plating method (Reed *et al.*, 2003), with incubation for 18-24 hours at 37°C.

2.1.2 Preparation of cell suspensions

All experimental cultures were prepared by loop inoculation of 10-200 mL of sterile nutrient broth (Oxoid, Basingstoke, UK or Labchem Himedia, Mumbai, India) for *E. coli* strains and brain heart infusion broth (Merck VWR, Darmstadt, Germany or Labchem Himedia, Mumbai, India) for *E. faecalis* strains in appropriate bottles or flasks. These broths were incubated overnight (18 h) at 37°C without shaking where the cells reached stationary phase in an environment that was essentially anaerobic, due to respiratory oxygen consumption (Khaengraeng and Reed, 2005).

Overnight broth cultures were centrifuged at room temperature at 12000 x *g* for 1 minute (MSE Microcentaur, Fisher Scientific, Loughborough, UK and Sigma AK15 Benchtop, Bangalore, India) for small volumes of cell suspension (1-2 mL) or centrifuged (Sigma, Laborzentrifugen, Germany) at 4°C at 5300 x *g* for 5 minutes for large volumes (10-160 mL). The supernatant was discarded and the pellet was suspended in sterile quarter-strength Ringer's solution (adjusted to pH≈7), thoroughly mixed with a vortex mixer and re-centrifuged. This whole process was repeated twice to remove all traces of the original growth medium from the cells. Finally the cells were suspended in sterile distilled water (adjusted to pH≈7) to a final density of 10^7-10^8 colony forming units per millilitre (CFU mL^{-1}). The cell suspensions were then exposed to various stresses as explained in the relevant Chapters.

2.2 Water sampling and sources

Environmental water samples were taken using sterile containers from three rivers in Panjab (India) located in Doraha, Khamanon and Kajauli (Plate 2.1), while the River Tyne was selected in the experiments conducted in Newcastle (Plate 2.2). All samples were collected as instructed by Standard Methods for the Examination of Water and Wastewater (Clesceri *et al.*, 1998), between 8:00 am and 9:00 am to avoid overexposure to sunlight, stored in an icebox and transported in darkness either to the Microbiology Department of Panjab University, Chandigarh within 90 minutes (Indian samples) or within 15 minutes to the laboratories of the University of Northumbria (UK samples), for processing.

2.3 Experimental equipment

Dehydrated media was weighed using a Sartorius 2434 electronic balance, accurate to 0.1 mg (Sartorius Limited, Epson, UK). A laminar flow hood was used for drying the molten agar plates prior to use in the experiment. A UV-C cabinet for ≥ 30 minutes was used for disinfecting 96-well multititre plates (Tarson, Bangalore, India) used in most probable number (MPN) method and also for 500 mL plastic (PET) bottles that could not be autoclaved. Calibrated Gilson semi-automatic pipettors with sterile disposable tips (Gilson Pipetman® Medical Electronics, Villiers-le-Bel, France) were used for dispensing small volumes of samples whereas for larger volumes sterile 10 mL glass pipettes were used. A multichannel pipettor (Anachem Limited, Luton, UK) of 25-200 µL was used for inoculating samples and liquid medium in most probable number (MPN) multiwell experiments. For membrane filtration, a vacuum pump, a Gelman magnetic filter funnel (Gelman Sciences, Ann Arbor, Michigan, USA) and cellulose ester membrane filters (Pall-Corporation, Ann Arbor, Michigan, USA) were used throughout the study. For conventional most probable number assays (MPN), 10 mL glass tubes and Durham tubes were used.

Plate 2.1 Map of Punjab displaying location of three sampling sites (Doraha, Khamanon and Kajauli)

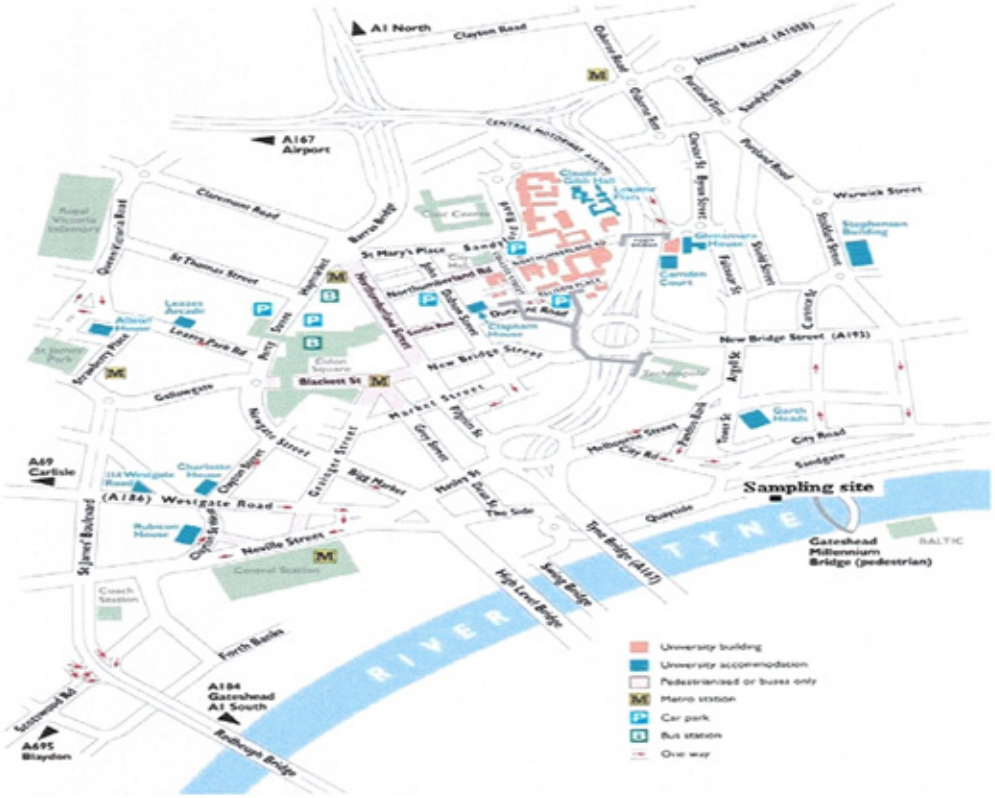


Plate 2.2 Map of Newcastle upon Tyne displaying location of sampling site (River Tyne)

2.4 Culture media and enumeration

2.4.1 Culture media

All media were obtained commercially in dehydrated form and prepared according to the manufacturer's instructions given on each product. Sufficient quantities of culture media in g L^{-1} to obtain triplicate culture plates for each sample were weighed and added to distilled water at 25°C. Culture media were often prepared in two sets; with and without the incorporation of a specific peroxide quencher of reactive oxygen species (ROS), such as sodium pyruvate at 0.05% w/v (0.5 g L^{-1}). The autoclaved media were poured into sterile 90 mm Petri plates and weighed using a top-pan balance to approximately 24 g, thus following Standard Methods for the Examination of Water and Wastewater (Clesceri *et al.*, 1998).

Media used for *E. coli* enumeration (with abbreviations) included nutrient broth, nutrient agar (N) and MacConkey agar (Mac) (Oxoid, Basingstoke,UK), Chromocult agar (CC), m-Lauryl sulphate agar (mLSA), m-Faecal Coliform agar without rosolic acid (mFC-R), and m-Endo (mEndo) (Merck, Darmstadt or Labchem Himedia, Mumbai, India). The media were prepared in the quantities given below:

- **Nutrient broth**: 25 g of the powdered medium was suspended in 1 L distilled water and then autoclaved at 121 °C for 15 min.
- **Nutrient agar:** 28 g of the powdered medium was suspended in 1 L distilled water and then autoclaved at 121 °C for 15 min.
- **Chromocult agar:** 26.5 g of the powdered medium was suspended in 1 L distilled water and then autoclaved at 121 °C for 15 min.
- **MacConkey agar:** 52 g of the powdered medium was suspended in 1 L distilled water and then autoclaved at 121 °C for 15 min.

- **Membrane faecal coliform agar without rosolic acid:** 52 g of the powdered medium was suspended in 1 L of distilled water and heated to boiling with frequent stirring until it was completely dissolved. Heating was continued for 1 min, with frequent agitation.
- **mEndo agar:** 51 g of the powdered medium was suspended in 1 L distilled water containing 20 mL of ethanol 96 % and heated to boiling, with frequent stirring until it was completely dissolved.
- **Membrane lauryl sulphate agar:** 76.2 g of the lauryl sulphate broth and 20 g of bacteriological agar was suspended in 1 L distilled water and then autoclaved at 121 °C for 15 min.

Table 2.1 Typical components of non-selective & selective media*
***Modified from Khaengraeng (2004)**

Abbreviation	Full name	Selective agents	Recommended by:
N	Nutrient agar	No selective ingredients	Use for comparative purposes
CC	Chromocult Coliform agar	Tergitol® 7	Manafi, 1998;
Mac	MacConkey agar (standard medium)	Neutral red Bile salts Crystal violet	WHO, 1963; Anon, 2002
mFC-R	Membrane Faecal Coliform agar without rosolic acid	Methyl blue (=aniline blue) Bile salts	Clesceri *et al.*, 1998
mEndo	m-Endo agar	Lauryl sulphate Deoxycholate Pararosaniline (=fuchsin)	Clesceri *et al.*, 1998
mLSA	Membrane lauryl sulphate	Lauryl sulphate	Anon, 2002

Chromocult Coliform agar (CC) allows simultaneous detection of both, *E. coli* and total coliforms in water and food samples, due to the development of distinct colony colouration as its formulation consists of two chromogenic substrates i.e. Salmon-galactoside and X-glucuronide which imparts a red colour to total coliforms and a purple colour to *E. coli* (red + blue = purple). The medium has a surfactant (Tergitol® 7) which inhibits the growth of Gram-positive bacteria as well as some Gram-negative bacteria (Geissler *et al.*, 1998). This medium also contains sodium pyruvate for the recovery of sub-lethally damaged bacteria.

MacConkey agar (Mac) was the medium recommended in 1958 for the bacteriological examination of water, and subsequently also by the World Health Organization (WHO, 1963) and the UK Department of Health (Anon., 1982). This medium contains bile salts as the inhibitory agent for Gram-positive bacteria and includes neutral red as a selective dye which imparts purple colour to organisms which are lactose fermenters.

Membrane faecal coliform agar (mFC-R), initially developed by Geldreich *et al.* (1965), is recommended by US standard methods (Clesceri *et al.*, 1998) for the detection of faecal coliforms by the membrane filtration technique, using an incubation temperature of 44.5°C ± 0.2 °C. Peptone and yeast extract serve as the nutritional source and bile salts are added to inhibit the accompanying Gram-positive flora. Lactose can be fermented by faecal coliforms at the elevated temperature to form blue colonies on the medium, whereas other organisms appear as grey colonies.

mEndo agar LES (mEndo) is a medium for the enumeration of total coliforms in water used by the standard membrane filter procedure in the US (Clesceri *et al.*, 1998). The Gram-positive bacteria are inhibited by the presence of sodium lauryl sulphate and deoxycholate while pararosanilin or fuchsin imparts a characteristic metallic sheen to the colonies.

Membrane lauryl sulphate agar (mLSA) contains sodium lauryl sulphate as the main inhibitory agent for Gram-positive bacteria and non-coliform micro-organisms. The UK standard methods (Anon., 2002) recommend this medium for the enumeration of coliform bacteria and *E. coli* in water with incubation at 30°C for 4 h then at 44°C for 14 h. Lactose-positive bacteria lower the pH and the indicator phenol red then gives a yellow colour to the colonies.

Media used for *E. faecalis* enumeration included brain heart infusion broth, nutrient agar (N), MacConkey agar no. 2 (M2), Bile aesculin agar (Bile), Slanetz and Bartley agar (SB), (Oxoid, Basingstoke, UK), KF Streptococcus agar (KF), (Merck VWR, Darmstadt). The media were prepared as described below:

- **MacConkey agar number 2:** 51.5 g of the powdered medium was suspended in 1 L distilled water and then autoclaved at 121°C for 15 minutes.
- **Bile aesculin agar:** 44.5 g of the powdered medium was suspended in 1 L distilled water and then autoclaved at 121°C for 15 minutes.
- **KF Streptococcus agar:** 71.5 g of the powdered medium was suspended in 1 L distilled water and heated to boiling with frequent stirring until it was

completely dissolved. A volume of 10 mL of 1% solution of TTC was added to the basal medium after boiling.

- **Slanetz and Bartley:** 42 g of the powdered medium was suspended in 1 L distilled water and heated to boiling with frequent stirring until it was completely dissolved.

MacConkey number 2 is a more selective modification of standard MacConkey agar and is suitable for the detection and enumeration of faecal enterococci (Bridson, 1998; Khaengraeng, 2004). The main inhibitory agent for Gram-negative bacteria is bile salts number 2. It contains neutral red in addition to crystal violet as acid producing dyes.

Bile aesculin azide agar introduced by Isenberg *et al.* (1970), contains azide as the selective agent and the aesculin as main diagnostic agent that reacts with iron to form a dark black coloured complex which imparts a halo-like boundary to *Enterococcus* colonies after 24 h at 35 ± 0.5°C in US (Clesceri *et al.*, 1998) or 44°C (UK standard methods, Anon, 2002). It is recommended by UK and US standard methods as a confirmatory medium.

KF streptococcus agar, initially introduced by Kenner *et al.* (1961), contains sodium azide as the main selective agent for inhibiting the growth of Gram- negative bacteria. Enterococci reduce triphenyltetrazolium chloride (TTC) to a insoluble red formazan which imparts a red colour to the colonies at an incubation temperature of 37°C after 48 h. Selective dyes such as bromocresol purple confirm acid production by changing its colour to yellow.

Slanetz and Bartley agar also sometimes called membrane-*Enterococcus* agar was initially introduced by Slanetz and Bartley (1957) and is recommended as a membrane filtration agar by UK and US standard methods. It contains sodium azide to inhibit the growth of Gram-negative bacteria. It contains triphenyltetrazolium chloride (TTC) that is reduced by enterococci after incubation for 48 h at 35 ± 0.5°C in US (Clesceri *et al.*, 1998) or 37°C in UK (Anon., 2002) to give an insoluble red formazan, producing red, maroon or pink colonies.

2.4.2 Enumeration of bacteria using culture-based methods

Timed samples were taken using suspensions of the defined strains of bacteria listed in section 2.1.1 in the case of laboratory-based experiments and from source waters in the case of field-based experiments. The small volumes of samples in laboratory studies were processed by serial decimal dilution in quarter-strength Ringer's solution to cover the dilution range between 10^0-10^{-5}. Triplicate plates were prepared for each sample using 0.02-1.0 mL of each dilution per sample using either, (i) the Miles and Misra surface droplet method for small volumes, or (ii) full spread plates for larger volumes (Reed, 1997; Khaengraeng, 2004). Plates were then incubated for 24 h under standard aerobic conditions, or for 48 h under anaerobic conditions obtained either (i) by using an Anaerocult® A sachet in an anaerobic jar of 2.5 L capacity (Merck Anaerocult® system; Anon. 2000) at 37°C in a conventional incubator (model MIR-153, Sanyo, Osaka, Japan) or (ii) by using a "Bugbox" anaerobic cabinet (Biotrace, Fred Baker, Bridgend, Wales) maintained at 37°C, containing an anaerobic gas mixture made up to 100% by using 5-10% H_2: 10% CO_2: 80% N_2. After 48 h incubation, the triplicate plates were examined for positive colonies, counted manually or using a colony counter (Stuart Scientific, Redhill, UK)

and further incubated for up to 72 h under aerobic conditions to confirm that no additional colonies had grown, before being discarded.

The counted colonies were expressed as CFU mL^{-1} by correcting for dilution and volume using the following equation (Reed *et al.*, 2003):

(CFU mL^{-1}) Colony forming units per millilitre = (C/V) x M (Eqn. 2.1)

Where C = colony count per plate

V = volume of sample inoculated on each plate (mL)

M = multiplication factor (reciprocal of the dilution used)

For example, if 40 colony forming units (CFU) are counted from an agar plate for a sample volume of 0.02 mL at a sample dilution of 10^{-2}, using equation 2.1, the colony count (CFU mL^{-1}) would be calculated as:

$(40/0.02) \times 10^{-2} = 2 \times 10^{5}$ CFU mL^{-1}

Plate counts were performed in triplicates and CFU mL^{-1} are shown in graphical format as geometric means based on log-transformed data with 95% upper and lower confidence limits where this transformation was used to normalize the data (Anon., 2002) and then converted back to non-log numerical format for ease of visual comparison. For example, log CFU mL^{-1} values of 6.3, 6.26 and 6.23 give an average of 6.26 with a 95% confidence interval of 0.089 converted back to a non-log number by using antilog of 6.26 which comes out to be 1.82×10^{6}. The numerical values for upper and lower 95% confidence limits were calculated by adding 6.26 and 0.089 i.e.

with 2.3 x 10^6 as the upper limit and 1.48 x 10^6 as the lower limit. This was calculated by anti-logging the value to obtain upper 95% confidence limit and subtracting the values to obtain lower 95% confidence limit. Comparisons between two different mean values can be based on overlap or non-overlap of their respective 95% confidence limits – thus, two means with non-overlapping 95% confidence limits can be regarded as different at the 95% level of probability, whereas two means with overlapping 95% confidence limits cannot. An alternative approach is to use the student's *t*-test for comparing two means, though this is laborious when several comparisons are made.

Plate counts of the larger volumes of natural water sample were processed by standard bacteriological membrane filtration (Clesceri *et al.*, 1998) using 1.0-100 mL of water filtered through 47 mm diameter Pall-Gelman filter membranes of 0.45 μm pore size. The filters were placed on the agar plates containing the growth medium by rolling the filter onto the medium carefully to avoid any air bubbles becoming trapped, and then incubated at 37°C under the conditions described above for the surface spread plates. The number of counted colonies on each plate (typically 20-100) was then expressed per 100 mL by multiplying the colony count (CFU) obtained by membrane filtration by 100 and dividing by the sample volume in mL (Clesceri *et al.*, 1998) using the following equation:

$$\text{CFU/100mL} = \frac{\text{number of colonies on membrane filter x 100}}{\text{volume of sample filtered (mL)}} \qquad \text{(Eqn. 2.2)}$$

For example, if 50 colony-forming units (CFU) were counted from the agar plate for a sample volume of 10 mL filtered, using equation 2.2, the colony count or CFU per 100 mL would be:

50 x 100/10 = 500 CFU per 100 mL

For standard MPN, a broth-based assay used 5 tubes containing lactose broth which were inoculated by sample volumes of 10, 1 and 0.1 mL representing serial ten-fold dilutions. The results were calculated by recording the number of positive tubes out of 5 tubes in each of 3 volumes, which are matched by using an MPN table to give the probable number of coliforms per 100 mL. For example, if 4 tubes in 10 ml volume, 4 tubes in 1 ml volume, and 2 tubes in 1 ml volume gave a positive result, then the MPN value per 100 mL would be 47 /100 ml.

The miniaturised MPN procedure of Maul and Block (1983), modified by Hernandez *et al.* (1991a), utilising 96- well multiwell (ELISA) plates was used to evaluate the novel broth-based media outlined in Chapters 6 and 7. The multiwell MPN approach provides more statistical precision to the MPN than the conventional 15-tube assay described above, and given narrower 95% confidence limits. However, its use is limited to smaller volumes of samples, typically 100-200 µL.

The multiwell plate used in the present study is a 96-well round bottom type (Plate 2.3). The multiwell plate contains a matrix of 12 x 8 wells used as 8 replicates (A to H) for 12 dilutions (1 to 12). Each 12 x 8 multiwell plate was used for a single sample, preparing five serial four-fold dilutions with 16 replicates by using duplicate columns for each dilution (2 x 8). In the first and seventh columns, 200 µL of undiluted sample was added using an Anachem multipipettor (Anachem, Luton, Bedfordshire, United Kingdom). To the remaining rows (2-6 and 8-12) was added 150 µL of sterile quarter-strength Ringer's solution as a diluent. The first four-fold dilution was prepared in rows 2 and 8 by transferring 50 µL of undiluted sample from the adjacent well (row 1 and 7), and then fully mixing using the multipipettor. This was repeated with the subsequent rows, to the fifth four-fold dilution, from

which 50 µL was then discarded, leaving 150 µL in each well across the plate. A volume of 100 µL of 250% strength broth medium of the appropriate composition was then pipetted carefully into each well without cross-contamination. Lids were then added and the plates incubated at 37°C for 48 h.

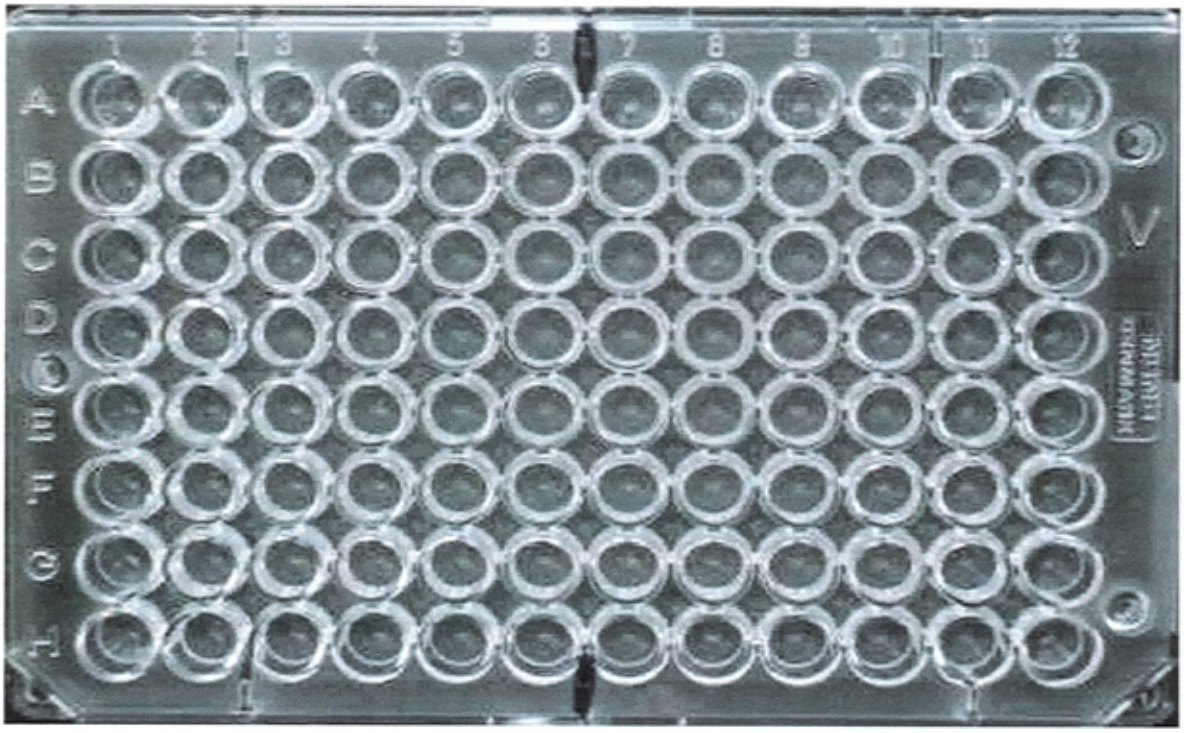


Plate 2.3 Photograph of 96-well mutliwell plate showing matrix of wells

The MPN calculator software (Curiale, 2004) was employed to determine the MPN values per mL, based on the number of positive wells for each dilution in the 96-multiwell plates. The MPN calculator provides an MPN value with 95% confidence limits per mL of each water sample tested. As an example, if the number of positives in order of dilution was 16 (undiluted sample), 16 (first four-fold dilution), 7 (second dilution), 0 (third dilution), 0 (fourth dilution) and 0 (fifth dilution) then MPN calculator gives an MPN mL^{-1} of 66 with a lower 95% confidence limit of 42 and an upper 95% confidence limit of 100.

Chapter 3

Comparative inactivation of faecal indicator bacteria in traditional brass and earthern water storage vessels*

* Components of this Chapter have been published in:

Antonie van Leeuwenhoek (2005) Volume 88, pages: 35-48.

3.1 Introduction

Recent studies of practical interventions to create safe water have clearly demonstrated that improved quality of water at the family level can have a significant impact in reducing water-borne diarrhoeal diseases (e.g. Quick *et al.*, 1996; Chidavenzi *et al.*, 1998; Wright *et al.*, 2004). In developing countries, in addition to the unsafe water collected for drinking purposes from contaminated sources, the situation is more complicated: water is often stored at home in various types of vessels, due to the infrequent availability of water, and this can lead to point-of-use contamination (Quick *et al.*, 2002; Reller *et al.*, 2003). The storage of water at the household level has been associated with evidence of increased contamination, depending upon a number of factors including the site of storage, the type of container used and post-collection handling practices (Kehoe and Conroy, 2002; Souter *et al.*, 2003; Trevett *et al.*, 2004).

Many of the small-scale methods to purify water and make it safe for drinking and other household purposes can be traced back to ancient times (Heber, 1985). The earliest recorded knowledge of water treatment is in the Sanskrit medical lore and Egyptian wall inscriptions (Shiva and Vandana, 1998). Thus Sanskrit writings, dating to around 2000 BC, describe how to purify water by storage in copper or brass vessels, by exposure to sunlight, or by filtering through charcoal and cooling in an earthen vessel (Patwardhan 1990; Reed, 2004). Although ancient people would not have been aware of how these treatments might have improved the microbiological quality of their water, they clearly appreciated the benefits brought by such methods in terms of their enhanced health and well-being (Baker and Taras, 1981; WHO, 2002a; Reed *et al.*, 2005).

In several parts of rural India, including Rajasthan (a water-scarce region) and Panjab (where water is plentiful), people continue to use brass and copper vessels ('mutkas') to carry and store water and there is a general belief among the rural people that such metal containers have beneficial properties against the agents of water-borne disease, helping to improve water quality by reducing the incidence of gastro-enteritis (Sharma and Sinha, 1993; WHO, 2002a; Reed *et al.*, 2005). The technologies that rural women have used for water purification are based on locally available products and traditional knowledge, such as the use of "balu mitti" (fine sand) to decrease the levels of turbidity and suspended solids in surface water prior to drinking (Reed *et al.*, 2005). When brass vessels were used for collection and storage, they were maintained regularly by cleaning/polishing and sometimes by coating internally with metals such as lead, copper and zinc in the Southern part of India, or tin in Northern India (Brick *et al.,* 2004). This was carried out by the local "kalliawala", though this has now become a rare practice (Tannar, 1998). A decline in the use of brass vessels, possibly because of their high cost, has led to an increasing number of households in which other types of storage vessels such as earthern, aluminium, stainless steel and plastic or untinned metal vessels are used (Brick *et al.,* 2004).

Since the earliest microbiological investigations of drinking water quality, the detection of faecal indicator bacteria, e.g. thermotolerant (faecal) coliforms or faecal streptococci (enterococci), in drinking water has been used as a means of predicting the possible presence of pathogenic bacteria (Chapter 1, Section 1.7). Culture-based microbiological techniques have made it possible to detect these indicator bacteria and other microbes in natural waters. This approach has been used to investigate the microbiological quality of water samples taken from various traditional storage vessels such as clay jugs ('zirs',

Khairy, 1982 or "mutkas", Phadke *et al.*, 1967) located in homes and at public water stands ('seebels', Hammad 1982), or from narrow-mouthed storage vessels (Mintz *et al.*, 1995; Crump *et al.*, 2004). Microbiological contamination at the household level, with the formation of biofilms in PVC water storage containers harbouring heterotropic bacteria along with total coliforms and *Clostridium perfringens*, has also been studied (Percival *et al.*, 2000; Jagals *et al.*, 2003; Trevett *et al.*, 2004).

Metals such as copper and its alloys e.g. brass (typically 2:1 w/w copper:zinc) exert their antimicrobial effect by interference with essential biological systems, e.g. by altering the three-dimensional structure of enzymes and other biomolecules, leading to death (Hassen *et al.*, 1998). For example, one action of copper is to oxidise the sulphydryl groups of amino acid residues within proteins, thereby inhibiting enzymatic activity (Yahya *et al.,* 1990). Recent research on the potential application of copper surfaces in food microbiology has demonstrated the inactivation of pathogens such as *Escherichia coli* O157, which failed to grow following 4 h exposure to copper surfaces at 18° C (Keevil, 2001; Faundez *et al.*, 2004). Such studies have mostly been carried out using either non-selective or selective agar-based media, incubated under standard aerobic conditions. However, recent studies on heat-stressed *Salmonella* (Stephens *et al.*, 2000) and *E. coli* O157 (Mizunoe *et al.*, 1999) have employed quenchers of reactive oxygen species (ROS) in the growth medium to demonstrate that ROS, derived mainly from aerobic respiration and auto-oxidation of medium constituents, may cause the inactivation of sub-lethally damaged cells of bacteria, thereby preventing their growth and enumeration under standard aerobic conditions (see also Chapter 1, section 1.9). The enhanced growth of sunlight-damaged *E. coli* under ROS-neutralised conditions (anaerobic incubation in a medium supplemented with the peroxide-scavenger sodium

pyruvate at 0.05% w/v) has also been noted recently (Khaengraeng and Reed, 2005), adding further support to the notion that ROS may adversely affect the counts of injured bacteria under conventional aerobic conditions. The present Chapter describes experiments carried out to investigate the extent of inactivation and injury of faecal indicator bacteria resulting from storage in brass and earthern vessels, with particular reference to the effects of (i) ROS-neutralising treatments and (ii) selective media on surface plate counts.

The specific objectives of the studies described in this Chapter were:

1. To investigate the effect of keeping water contaminated with different strains of *Escherichia coli* in traditional brass or earthern vessels (mutkas) of the type used to collect and store water in rural India, with enumeration by surface plate counts on a non-selective medium (nutrient agar) under standard aerobic conditions or under conditions designed to neutralise reactive oxygen species.
2. To compare the enumeration of *E. coli* kept in water in a brass vessel and then plated onto non-selective nutrient-rich and low-nutrient media, and onto different selective media to investigate the effects of the nutrient status and selectivity of the medium on bacterial counts.
3. To study the effect of storage of contaminated water from natural sources, along with samples of water collected directly from traditional vessels in households in rural Panjab on the enumeration of coliform bacteria using various selective agar media and under aerobic and ROS-neutralised conditions.
4. To compare different strains of *E. faecalis* kept in water in a brass vessel and then counted on various selective media in comparison with a non-selective medium under aerobic and ROS- neutralised conditions.

3.2 Materials and Methods

Cell suspensions of bacterial cultures were prepared as described in Chapter 2. Cells were suspended in sterile distilled water, with the pH adjusted to ≈ 7 using dilute sodium hydroxide solution, to give an overall dilution of 1:100, i.e. 160 mL of broth was used to prepare 16 L of suspension.

Storage vessels Brass and earthern mutkas (12 L capacity) were obtained from local merchants in Doraha, Panjab, India. Before use, they were scrubbed thoroughly with a non-abrasive cloth to remove any adherent microflora, disinfected using 0.1% w/v Virkon, rinsed 3 times with sterile distilled water, then soaked for at least 3 h and re-rinsed 3 times, to remove all traces of disinfectant. In some experiments, plastic, stainless steel, copper or glass vessels of equivalent volumes were also used, for comparison. Plate 3.1 shows example of some of the different types of mutkas in use across India.

3.2.1 Effects of ROS-neutralisation on the enumeration of *Escherichia coli* NCTC8912 from water kept in various storage vessels

Laboratory experiments were carried out using *E. coli* NCTC8912 cells grown in nutrient broth as detailed in Chapter 2. The cell suspension in sterile distilled water (16 L) was poured equally (8 L) into (i) a brass mutka and (ii) an earthern mutka, and then kept at 25°C. Triplicate samples were taken at 0, 6, 24, and 48 h and processed by serial decimal dilution then surface spread plated using the Miles and Misra droplet method described in Chapter 2.

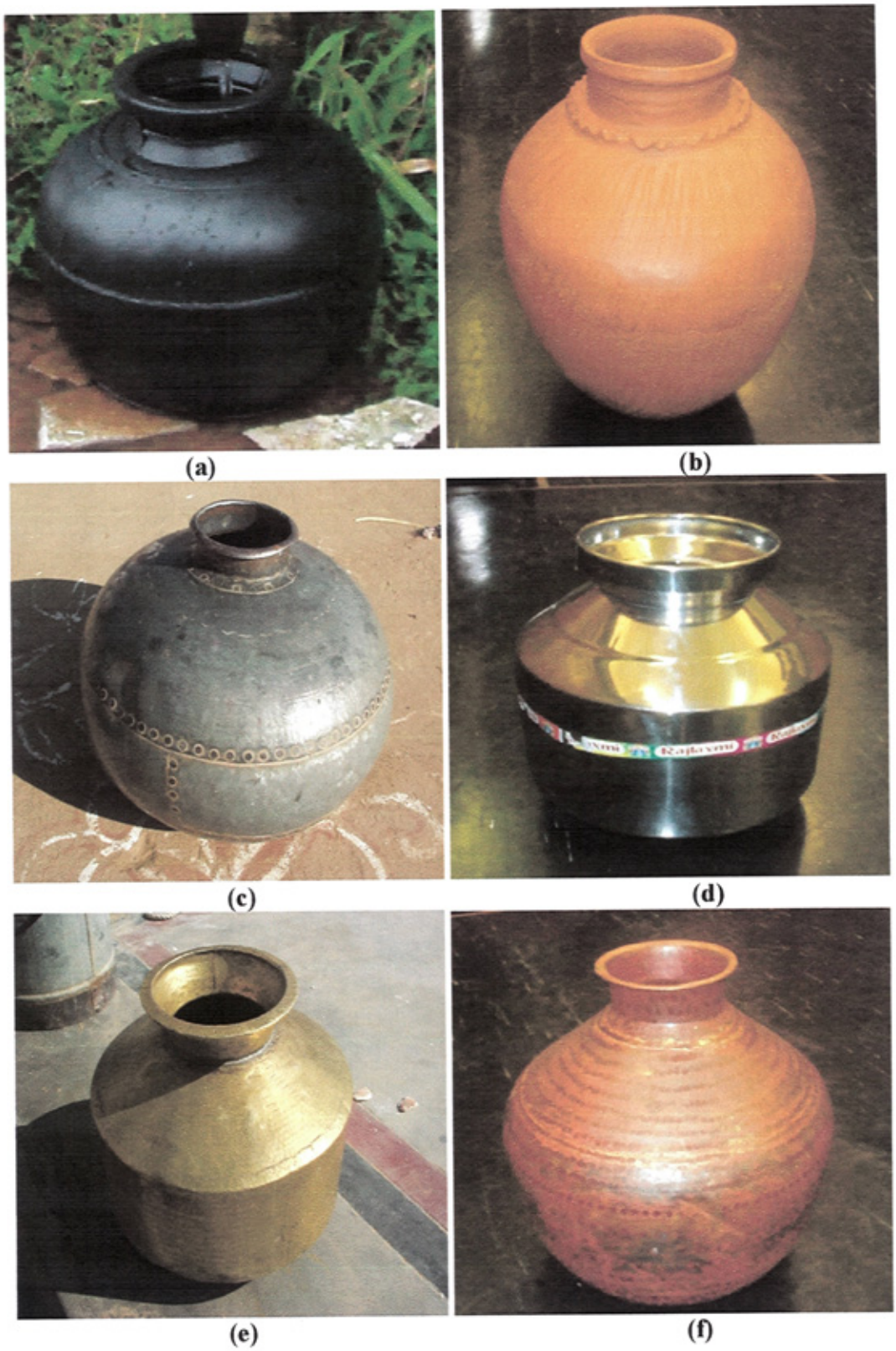


Plate 3.1 Various storage containers from India (a) plastic vessel, (b) earthern vessel, (c) cast iron vessel, (d) stainless steel vessel, (e) brass vessel, (f) copper vessel.

Plates with and without supplementation of the growth medium with 0.05% w/v (0.5 g L^{-1}) sodium pyruvate as a specific neutralising agent for peroxides (MacDonald *et al.*, 1983; Curtis *et al.*, 1992; Khaengraeng and Reed, 2005) were incubated either (i) aerobically, (ii) anaerobically in a 2.5 litre anaerobic jar at 37°C in a conventional incubator, (iii) at 37°C in an anaerobic cabinet, or (iv) at 37°C in an anaerobic cabinet on pre-reduced medium, where the plates were kept for 24 h in the anaerobic cabinet prior to use (Chapter 2), and inoculated within the cabinet, to maintain fully anaerobic conditions from the start of the experiment.

3.2.2 Effects of low redox potential on enumeration of *Escherichia coli* NCTC8912 from water kept in a brass vessel

To investigate the possible effects of low redox potential on enumeration, a sample of *E. coli* NCTC8912 was taken at 0 h and after 6 h in water kept in a brass vessel and enumerated on nutrient agar with and without 0.05% w/v sodium pyruvate. Two sets of plates were made for each medium: 10 mM dithiothreitol (DTT) stock solution was freshly prepared and 200 µL was spread on the surface of one set of agar plates while the other set was kept without DTT. DTT is known to decrease the redox potential of the medium (George and Peck, 1998). The samples of *E. coli* were diluted and surface spread onto both sets of agar plates and incubated under aerobic and anaerobic conditions in triplicate for all four types of conditions.

3.2.3 Effects of carry-over of the storage water on enumeration of *Escherichia coli* NCTC8912 from water kept in a brass vessel

An *E. coli* NCTC8912 sample was taken at 0 h (initial inoculum) and after 6 h suspension in water kept in a brass mutka. The 6 h sample was divided into two sets: the

first set was centrifuged at 5000 x *g* for 10 minutes, rinsed and recentrifuged and the second set was enumerated directly. This approach was used to establish whether the copper and zinc metal ions dissolved in the 6 h water sample might decrease the colony count of *E. coli* during the enumeration process as a result of carry-over in the dilution and plating procedure. Both sets of plates were enumerated using non-selective nutrient agar with and without 0.05% w/v sodium pyruvate supplementation under aerobic and anaerobic conditions.

3.2.4 Effects of the nutrient status of the enumeration medium on counts of *Escherichia coli* NCTC8912 from water kept in a brass vessel

Experiments were carried out using *E. coli* NCTC8912 cells suspended in water kept in a brass mutka for 6 h. Samples were surface spread onto the following media, prepared with and without added sodium pyruvate at 0.05% w/v:

(i) conventional nutrient agar (N), a standard nutrient medium used for routine culture of non-fastidious bacteria,

(ii) R2A (Reasoner and Geldrich, 1985), a low-nutrient 'minimal' medium used for maximum recovery of stressed bacteria (note that while it was prepared here in pyruvate-free and pyruvate-supplemented formats this medium usually contains sodium pyruvate as one of its constituents in the commercial format);

(iii) brain-heart agar (BA), a nutrient-enriched medium often used for more fastidious bacteria (Bridson, 1998).

Nutrient agar was prepared from the commercial product (Oxoid, Basingstoke, UK), at 25 g L^{-1} according to the manufacturer's instructions (Chapter 2). Pyruvate-free R2A medium was prepared from individual constituents, as follows: 0.5 g yeast extract, 0.5 g proteose peptone, 0.5 g casein hydrolysate, 0.5 g glucose and 0.5 g

soluble starch, 0.3 g di-potassium hydrogen phosphate, 0.05 g magnesium sulphate (all Sigma-Aldrich, Gillingham, UK) and 12 g bacteriological agar (Oxoid, Basingstoke, UK), dissolved in 1 L of distilled water. Brain-heart agar was prepared from the commercial product (Merck VWR, Darmstadt, Germany), at 52 g L^{-1}. Pyruvate-supplemented media were prepared at 0.05% w/v (0.5 g L^{-1} sodium pyruvate). All media were autoclaved at 121°C for 15 min. Inoculated media were incubated either (i) aerobically or (ii) anaerobically in a 2.5 litre anaerobic jar in a conventional incubator at 37°C or (iii) in an anaerobic cabinet maintained at 37°C.

3.2.5 Effects of medium composition and selective agents on different strains of *E. coli* from water kept in a brass vessel

Four different strains of *E. coli* (NCTC 8912, TN675, PUCC061 and PUCC113; Chapter 2) were stored in water kept in the brass and earthern mutkas for up to 48 h. Each strain was used to compare and investigate the effects of medium composition and selective agents. Here, the media used included non-selective nutrient agar medium, together with following selective media: MacConkey agar, m-lauryl sulphate agar (mLSA), mFC agar without rosolic acid (mFC-R) and mEndo agar. All of the above commercial media were prepared as detailed in Chapter 2. Media were prepared in sufficient quantities to allow counts to be performed in triplicate, with and without added sodium pyruvate at 0.05% w/v (0.5 g L^{-1}), with incubation under standard aerobic conditions and in an anaerobic jar.

3.2.6 Effects of enumeration conditions on coliform counts for environmental and household water samples

Environmental water samples Having established from the initial studies with pure cultures that ROS-neutralised conditions could be obtained using a combination of a growth medium with added sodium pyruvate (0.05% w/v) together with incubation in an anaerobic jar, aliquots of natural water samples from river sources located in Doraha, Khamanon and Kajauli (see Plate 2.1 for map) were processed by standard bacteriological membrane filtration procedures (Clesceri *et al.*, 1998) as described in Chapter 2 using various selective media (MacConkey, mFC-R, mEndo and mLSA) either (i) aerobically for media without added pyruvate (standard aerobic conditions) at 37°C or (ii) in an anaerobic jar at 37°C for media with added pyruvate (ROS-neutralised conditions) to provide initial counts for presumptive total coliforms (TC), prior to storage. Water (8 L) from each of the sources was then poured into the brass and earthen mutkas and incubated at 28°C, with sampling and enumeration by membrane filtration at timed intervals up to 48 h. All counts for natural water samples are expressed per 100 mL.

Household water samples Field samples were taken from brass and earthen mutkas used for drinking water storage within individual households in three rural locations in Panjab: (i) Gujrawala (source: hand pump); (ii) Doraha (source: municipal tap); and (iii) Sector 40, Chandigarh (source: municipal tap). These samples were collected at 8.00 am to 9.00 am from brass and earthern mutkas in each area, where the water had been collected the previous day and stored overnight. These samples, along with water obtained at the same time from each of the sources used to fill these vessels (and stored overnight in freezer prior to testing), were returned to the laboratory at Chandigarh

University within 90 min. Samples were processed by membrane filtration using the selective media described above, to provide an indication of the initial inoculum of presumptive total coliforms (TC) in the vessels to compare with the values obtained for the stored water from the mutkas following overnight incubation.

3.2.7 Effects of ROS-neutralisation on enumeration of *Enterococcus faecalis* NCTC775 from water kept in brass and earthern storage vessels

Timed samples of *E. faecalis* NCTC775 cell suspension were taken at 0 h (initial inoculum) and after 12 h (final inoculum) from water stored in the brass and earthern vessels. Enumeration of samples was carried out using non-selective nutrient agar with and without supplementation of 0.05% w/v sodium pyruvate. Plates were prepared in triplicate and incubated for 48 h at 37°C under standard aerobic conditions and under the various anaerobic conditions that have been described for *E. coli* in section 3.2.1 i.e. in an anaerobic jar, or in an anaerobic cabinet, or onto pre-reduced plates in an anaerobic cabinet.

3.2.8 Effects of the nutrient status of the enumeration medium on counts of *Enterococcus faecalis* NCTC775 from water kept in a brass vessel

The initial inoculum (0 h) and final sample after 12 h storage in water kept in a brass vessel were processed using three non-selective growth media of different nutrient status, as for *E. coli* namely, (i) nutrient agar, (ii) R2A agar and (iii) brain-heart agar with and without supplementation of the medium with 0.05% w/v sodium pyruvate. Plates were incubated at 37°C either under standard aerobic conditions or under anaerobic conditions (anaerobic jar).

3.2.9 Effects of medium composition and selective agents on different strains of *Enterococcus faecalis* from water kept in a brass vessel

Four different strains of *Enterococcus faecalis* (NCTC775, ATCC35550, PTO1 and PTO2; Chapter 2) were suspended in water kept in a brass vessel for up to 48 h. These strains were used to compare the effects of medium composition and selective agents on enumeration. The media used for *E. faecalis* included, non-selective nutrient agar (N), along with following selective media: MacConkey agar number 2 (M2), Bile aesculin agar (BAA), KF Streptococcus agar (KF) and Slanetz and Bartley agar (SB). Plates were prepared in triplicate as detailed in Chapter 2. Enumeration was carried out using all media with and without 0.05% w/v sodium pyruvate supplementation, with incubation for 48 h at 37°C under aerobic and anaerobic conditions.

3.3 Results

3.3.1 Effects of ROS-neutralisation on the enumeration of *Escherichia coli* NCTC8912 from water kept in various storage vessels

Plate counts are shown in Figure 3.1a for a suspension of *E. coli* NCTC8912 prior to storage (0 h), enumerated on nutrient agar in the presence and absence of 0.05% w/v sodium pyruvate either aerobically, or under one of three different sets of anaerobic condition. In the first two sets of anaerobic conditions, dilution and spread plating were carried out on the laboratory bench under aerobic conditions, the plates were then transferred either to an anaerobic jar, which took around one hour to create fully anaerobic conditions (Anon., 2000), or to an anaerobic cabinet, which provided a fully anaerobic atmosphere from the start. In the third set, dilutions were performed and anaerobic plates were prepared entirely within the anaerobic cabinet, with spread plating onto pre-reduced medium, with subsequent incubation in the same cabinet to provide anaerobic conditions throughout the experiment. The results indicate that at 0 h there was no discernible difference between the counts obtained under aerobic conditions, or in any of the anaerobic systems for nutrient agar medium either with or without added pyruvate (Figure 3.1a). Consequently, for most of the subsequent experiments with *E. coli* strains the initial inoculum was determined by taking the overall average of triplicate counts under (i) standard aerobic conditions using unsupplemented nutrient agar and (ii) anaerobic conditions (anaerobic jar) using in pyruvate-supplemented nutrient agar.

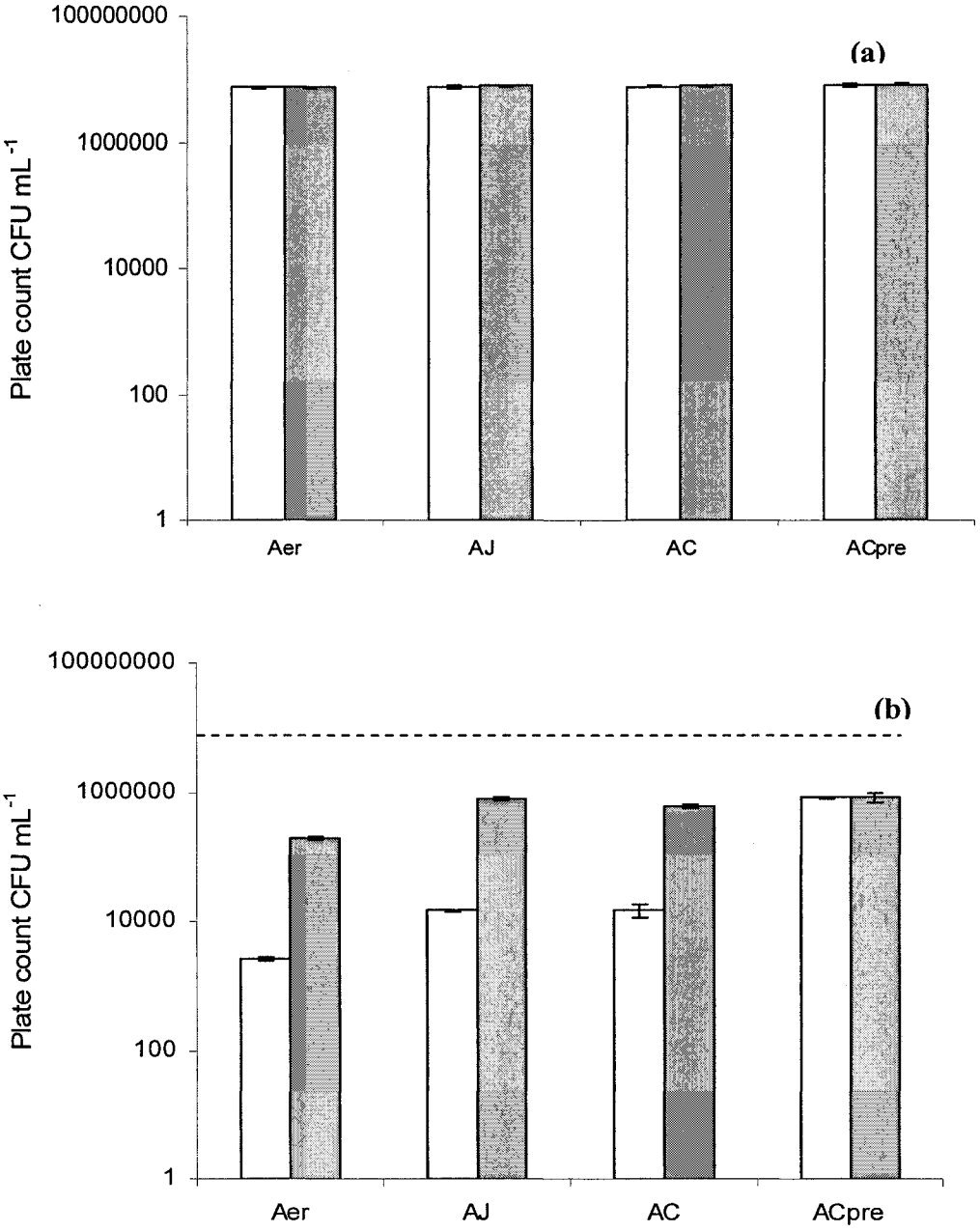


Fig. 3.1 Effects of ROS-neutralisation on the enumeration of *Escherichia coli* NCTC8912 from water kept in a brass vessel (a) at 0 h in sterile distilled water; (b) following 6 h storage in a brass mutka; enumerated on nutrient agar (unshaded bars) or on nutrient agar supplemented with 0.05% w/v sodium pyruvate (shaded bars) prepared and cultured under aerobic conditions (Aer), prepared under aerobic conditions and cultured either in an anaerobic jar (AJ) or in an anaerobic cabinet (AC), or prepared under anaerobic conditions using pre-reduced medium and maintained in an anaerobic cabinet (ACpre). The initial inoculum in (b) is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

In contrast, following 6 h incubation in the brass mutka, there were substantial decrease in counts compared to the corresponding initial values (Figure 3.1b), with the reduction varying from around 10-fold in real terms to over 1000-fold, depending upon the enumeration conditions (note that the vertical axis is log-transformed, to cover a wider range of counts on a single graph). Water stored in the brass vessel and then enumerated under aerobic conditions in pyruvate-unsupplemented medium showed the lowest mean count, with pyruvate-supplemented plates giving a 70-fold higher mean count than for unsupplemented medium under aerobic conditions ($P = 3.0 \times 10^{-7}$) . A similar pattern of higher mean counts for the pyruvate-supplemented medium was also observed for plates prepared in air and then transferred either to the anaerobic jar ($P = 2.3 \times 10^{-5}$) or to the anaerobic cabinet ($P = 5.6 \times 10^{-5}$): however, in each case the count obtained under anaerobic conditions was slightly greater, at around 4-fold higher than the count for the equivalent medium obtained under aerobic conditions. In contrast, no such difference in counts was obtained between pyruvate-supplemented and unsupplemented plates prepared and maintained in the anaerobic cabinet using pre-reduced medium (Figure 3.1b). Thus a count of approximately 10^6 CFU mL^{-1} was obtained for four sets of conditions, namely samples processed under aerobic conditions on pyruvate-supplemented medium which was then incubated either (i) in an anaerobic jar or (ii) an anaerobic cabinet, along with samples processed under anaerobic conditions and plated either onto pre-reduced (iii) pyruvate-supplemented or (iv) unsupplemented growth medium entirely within the anaerobic cabinet. These results indicate that the combination of an anaerobic jar and a pyruvate-supplemented medium can give an average count equivalent to that obtained when using pre-reduced unsupplemented medium ($P = 0.22$) or pyruvate-supplemented medium ($P = 0.35$) in an anaerobic cabinet.

Presumably, in all of these four sets of conditions, the damaging effects of ROS are neutralised and consequently, the term "ROS-neutralised conditions" is applied to such conditions hereafter. In all subsequent experiments in India, ROS-neutralised conditions were obtained by carrying out dilution and plating procedures in air on pyruvate-supplemented medium with subsequent incubation in an anaerobic jar, as an anaerobic cabinet was not available in the laboratory at Chandigarh. After 24 h incubation in the brass mutka, the stored water gave no detectable counts for any growth medium or incubation conditions.

Figure 3.2a shows equivalent data for the same suspension kept in the earthern mutka for 6 h. In contrast to the results for the brass vessel, storage in an earthern container resulted in a minimal change in count, with no substantial difference across any of the growth conditions used. Similarly, after 24 h incubation in the earthern mutka, there was minimal change in the count across the various growth conditions (Figure 3.2b).

Various storage vessels i.e. glass, stainless steel, plastic, earthern, copper and brass also were tested in the initial studies. The results shown in Table 3.1 demonstrate that *E. coli* suspensions stored in the copper vessel showed a similar pattern of decrease in counts after 6 h to that seen with the brass vessel while the stainless steel, glass and plastic storage vessels showed similar minimal decrease in counts at 6 h as was obtained using the earthern mutka. Table 3.1 also shows broadly similar enhancements in counts under peroxide-neutralised (pyruvate-supplemented), anaerobic (anaerobic jar) and ROS-neutralised conditions (pyruvate supplemented medium incubated in an anaerobic jar) for water stored in copper and brass vessels.

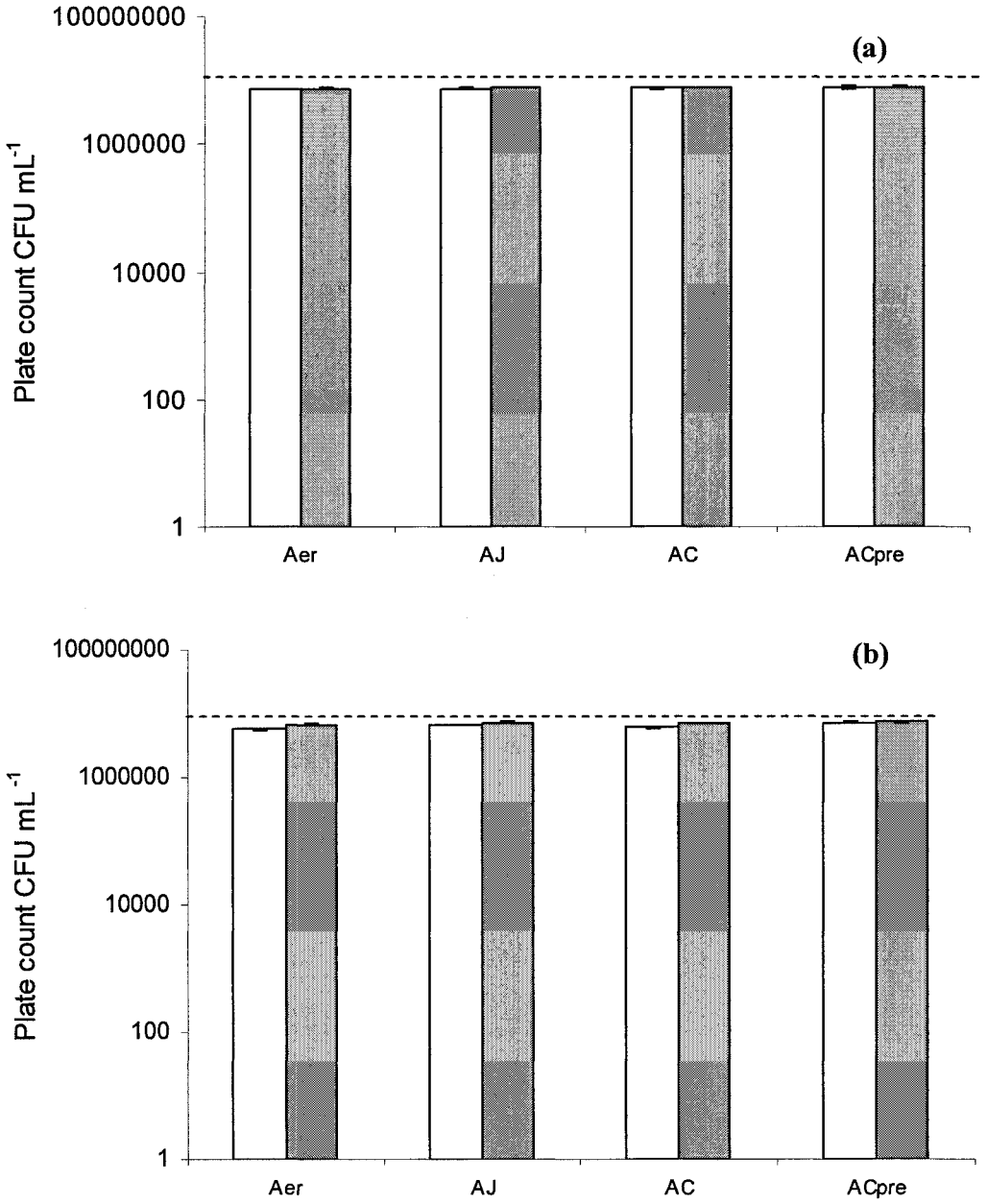


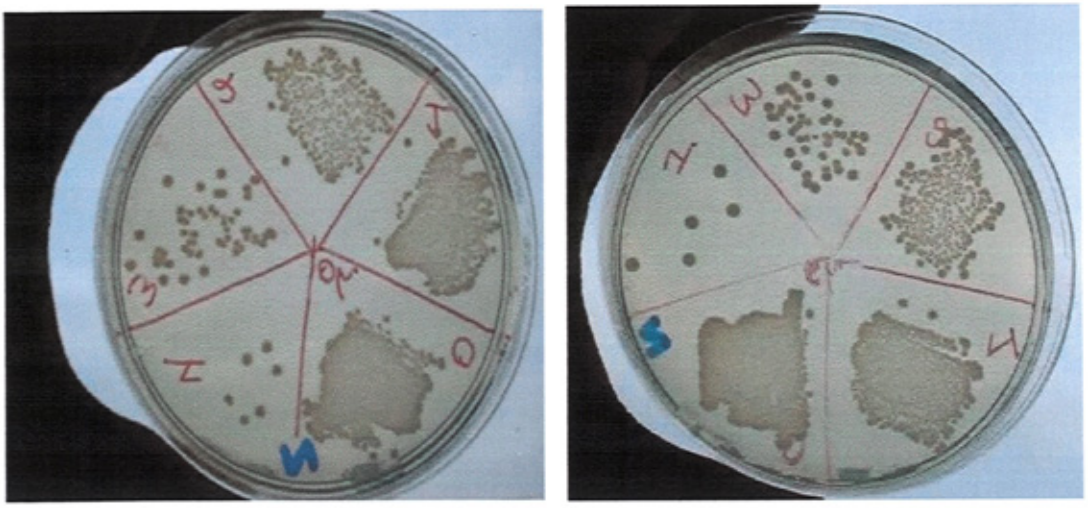
Fig. 3.2 Effects of ROS-neutralisation on the enumeration of *Escherichia coli* NCTC8912 from water kept in an earthern vessel (a) following 6 h storage in an earthern mutka ;(b) following 24 h storage in an earthern mutka, enumerated on nutrient agar (unshaded bars) or on nutrient agar supplemented with 0.05% w/v sodium pyruvate (shaded bars) prepared and cultured under aerobic conditions (Aer), prepared under aerobic conditions and cultured either in an anaerobic jar (AJ) or in an anaerobic cabinet (AC), or prepared under anaerobic conditions using pre-reduced medium and maintained in an anaerobic cabinet (ACpre). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Table 3.1 Effects of storage in water kept in different vessels on counts of a suspension of *Escherichia coli* NCTC 8912 Mean counts are shown (upper and lower 95% confidence limits in brackets below each mean value) for *E. coli* suspended for 0 h and 6 h, incubated either aerobically, or aerobically with supplementation of the medium with 0.05% w/v sodium pyruvate (peroxide-neutralised), anaerobically (anaerobic jar), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised).

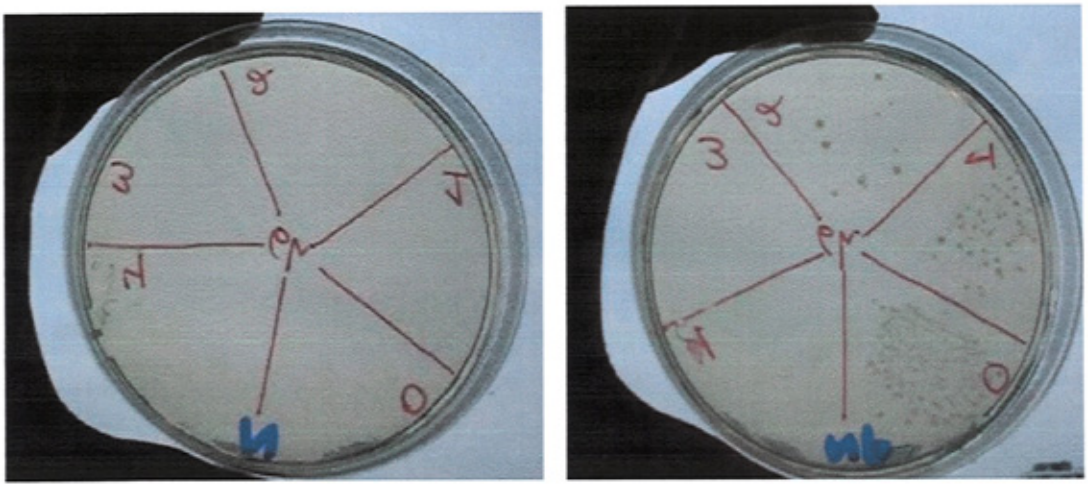
		Mean count (CFU mL^{-1})			
Vessels	**Time (h)**	**Aerobic**	**Peroxide-neutralised**	**Anaerobic**	**ROS-neutralised**
Initial inoculum	0	7.5×10^6 (6.4×10^6- 9.0×10^6)	8.0×10^6 (7×10^6- 9.0×10^6)	7.0×10^6 (5.0×10^6- 9.5×10^6)	7.4×10^6 (6.5×10^6- 8.5×10^6)
Glass	6	7.0×10^6 (6.0×10^6- 8.0×10^6)	7.6×10^6 (7×10^6-8.0×10^6)	7×10^6 (6.0×10^6- 8.4×10^6)	7.8×10^6 (7.2×10^6- 8.5×10^6)
Stainless steel	6	5.6×10^6 (4.5×10^6- 7.0×10^6)	7.0×10^6 (6.0×10^6-8.0×10^6)	6.0×10^6 (5.0×10^6- 7.0×10^6)	8.0×10^6 (6.5×10^6- 9.7×10^6)
Plastic	6	6.7×10^6 (6.0×10^6- 7.4×10^6)	7.0×10^6 (6.0×10^6-7.6×10^6)	6.8×10^6 (6.0×10^6- 7.4×10^6)	7.5×10^6 (6.6×10^6- 8.5×10^6)
Earthern	6	6.0×10^6 (5.4×10^6- 7.0×10^6)	6.6×10^6 (5.0×10^6-8.4×10^6)	6.0×10^6 (5.0×10^6- 6.5×10^6)	6.6×10^6 (5.0×10^6- 8.4×10^6)
Brass	6	6.0×10^2 (1.7×10^2- 2.0×10^3)	6.0×10^4 (5.0×10^4-7.6×10^4)	5.4×10^3 (4.5×10^3- 6.4×10^3)	3.6×10^5 (2.5×10^5- 5.0×10^5)
Copper	6	7.7×10^2 (2.3×10^2- 2.7×10^3)	7.0×10^4 (5.0×10^4-9.0×10^4)	4.3×10^3 (3.0×10^3- 6.2×10^3)	1.12×10^5 (5.0×10^4- 2.6×10^5)

An experiment was carried out to see whether the inactivation observed in the brass vessel was due to metal ions dissolving into the water or whether direct contact with the metal surfaces was required. An *E. coli* NCTC8912 cell suspension was inoculated into a glass flask filled with water that had been pre-stored in the brass mutka for 48 h and the results are shown visually in Plate 3.2. A decrease in the count was observed after 6 h incubation in the water that had been pre-stored in the brass mutka, but not in the control (no pre-storage).

For the pre-stored water incubated for 6 h, the decrease in the count enumerated on nutrient agar (initial inoculum = 4.0×10^6 CFU mL $^{-1}$) was noted to be around 80000-fold in the case of standard aerobic conditions (5.0×10^1 CFU mL $^{-1}$) while under ROS-neutralised conditions there was a decrease of just over 100-fold (3.0×10^4 CFU mL $^{-1}$). These results are broadly similar to the results shown in Fig. 3.1b for *E. coli* NCTC8912 suspended in water stored in the brass mutka for 6 h, indicating that copper/zinc present in the pre-stored water must be responsible for the observed effects in Plate 3.2. The overall dilution and droplet counting procedure is also visible in this set of photographs. Similarly a copper sulphate solution ($CuSO_4$ at 0.158 mg per Litre) was prepared in sterile autoclaved water (pH=7) and stored in a glass vessel. An *E. coli* cell suspension was inoculated and timed samples were taken. After 24 h the results showed a decrease in cell count compared with the initial inoculum (6.12×10^5 CFU mL $^{-1}$). The reduction in cell count varied under aerobic conditions (1.1×10^3 CFU mL $^{-1}$) and ROS-neutralised conditions (2.05×10^4 CFU mL $^{-1}$) in a similar manner to that shown in Fig. 3.1b. Thus the results were in agreement with those obtained for the *E. coli* cell suspension in water stored in the brass mutka.



(a) (b)



(c) (d)

Plate 3.2 Enumeration of *E. coli* NCTC8912 in water pre-stored in a brass vessel and transferred to a glass flask Miles and Misra droplet plates are shown for nutrient agar: (a) at 0 h, (b) at 6 h from control, (c) After 6 h in the pre-stored water, counted aerobically on unsupplemented medium, (d) After 6 h in the pre-stored water, counted under ROS-neutralised conditions (pyruvate-supplemented medium under anaerobic conditions).

In order to determine the amount of copper leached into solution within each type of vessel, distilled water (pH ≈ 7) was stored in the brass, copper and earthern vessels for 48 h. Inductively Coupled Plasma-Mass Spectrometry (X-Series ^{II} ICP-MS; Thermo Electron Corporation, Cheshire, UK) was then performed on a sample to measure the amount of copper ions dissolved in the water. The results obtained in Fig. 3.3 show that the levels of dissolved copper ions for water kept in the copper and brass vessels gave values of around 0.4 mg L^{-1} and 0.15 mg L^{-1}, while the amount in water kept in the earthern vessel remained undetectable even after 48 h storage time. The level of dissolved copper ions was less for the water which was stored for 48 h in the brass mutka, indicating that a smaller amount of leaching of copper occurs from the surface of the brass mutka in contrast to the higher value obtained for water stored for 48 h in the copper mutka. Presumably, this is due to the lower copper content of the brass vessel. Taken together with the results of Figure 3.1, Table 3.1 and Plate 3.2, this suggests that low levels of copper dissolved in the water are sufficient to inactivate *E. coli* over this time period.

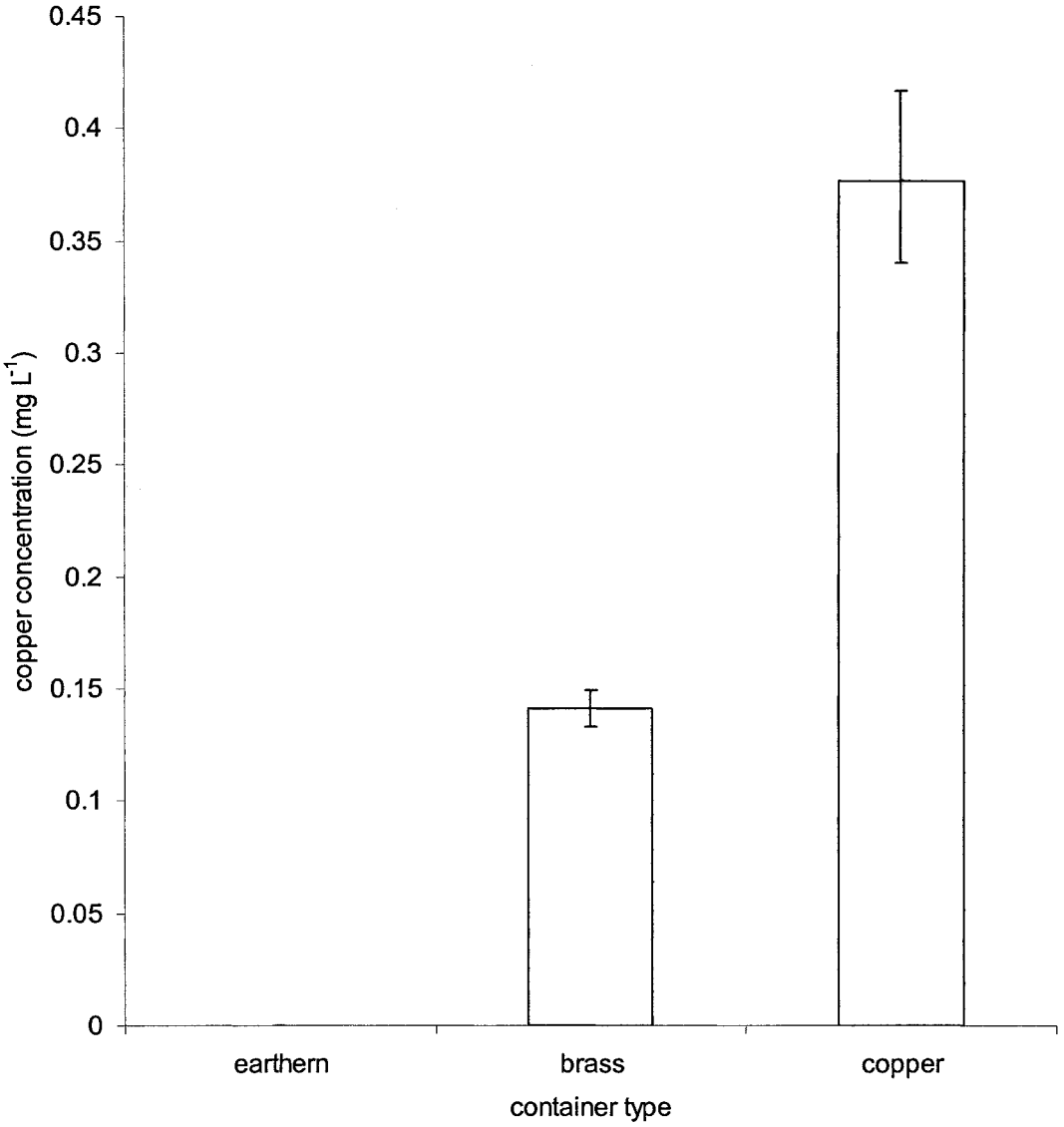


Fig. 3.3 Levels of copper ions dissolved in water stored in copper, brass and earthern vessels for 48 h measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

3.3.2 Effects of low redox potential on enumeration of *Escherichia coli* NCTC8912 from water kept in a brass vessel

Figure 3.4 shows plate counts for *E. coli* NCTC8912 after 6 h incubation in water kept in a brass mutka and enumerated onto nutrient agar with and without supplementation of 0.05% sodium pyruvate. The plates were incubated at 37°C either, (i) aerobically with and without DTT, or (ii) anaerobically with and without DTT (in an anaerobic jar). Substantial decreases in counts were seen when compared to the initial inoculum with the reduction varying from less than five-fold to over one thousand fold, depending upon the enumeration conditions. In the case of aerobic enumeration, addition of DTT to the standard nutrient agar unsupplemented medium increased the colony count by over 5-fold, whereas addition of DTT to the pyruvate-supplemented nutrient agar medium led to only an increase to just over double the count compared to the same medium without DTT. Anaerobic enumeration (anaerobic jar) using standard unsupplemented nutrient agar medium increased the colony count to almost 3-fold, compared to the aerobic count. However, addition of DTT to the pyruvate-supplemented nutrient agar medium showed no substantial effect. Thus the results clearly indicate that while DTT is effective in increasing the count on nutrient agar plates incubated aerobically and anaerobically in the absence of 0.05% sodium pyruvate by lowering the redox potential of the medium, in all other growth conditions i.e. in pyruvate-supplemented nutrient agar under aerobic conditions and anaerobic conditions the inclusion of DTT has no substantial beneficial effect on colony count.

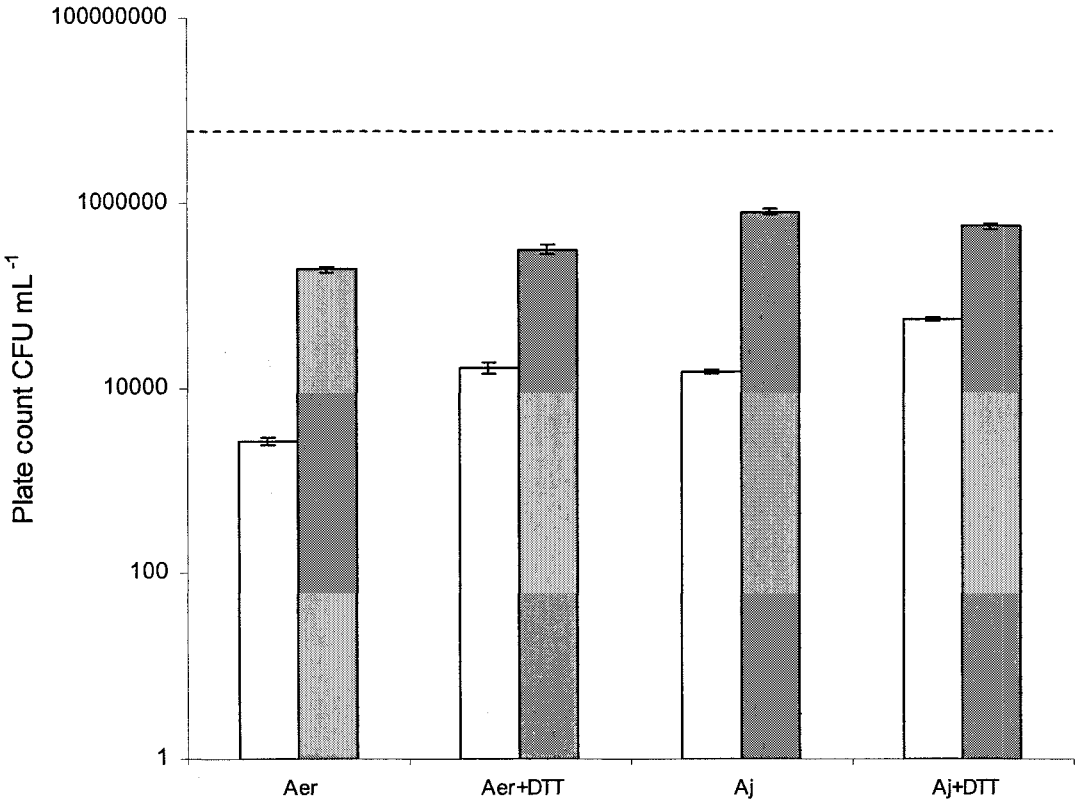


Fig. 3.4 Effects of low redox potential on enumeration of *Escherichia coli* NCTC8912 from water kept in a brass vessel following 6 h storage in water kept in a brass mutka, samples were enumerated on nutrient agar (unshaded bars) or on nutrient agar supplemented with 0.05% sodium pyruvate (shaded bars) prepared and cultured under either, (i) aerobic conditions (Aer), (ii) aerobic conditions + dithiothreitol (Aer + DTT), (iii) in an anaerobic jar (Aj), (iv) in an anaerobic jar + dithiothreitol (Aj + DTT). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). "Note that the vertical axis is log-transformed".

3.3.3 Effects of carry-over of the storage water on enumeration of *Escherichia coli* NCTC8912 from water kept in a brass vessel

Figure 3.5 shows data for *E. coli* NCTC8912 after 6 h suspension in water kept in a brass mutka, to find out the effects of carry-over of the incubation medium on the counts obtained. The results showed lower colony counts compared with the initial values: the broad trends of the results obtained on storage in a brass mutka in the three sets of enumeration conditions were similar to those obtained in Fig. 3.1b. Thus the aerobic unsupplemented medium showed the lowest count followed by anaerobic unsupplemented medium and then the pyruvate-supplemented medium under aerobic conditions. The pyruvate-supplemented medium in the anaerobic jar and pre-reduced medium in an anaerobic cabinet showed the highest count. The differences in count obtained where the initial sample was diluted with and without any carry-over were very small and were most clearly seen for aerobic unsupplemented medium. The differences observed for all other conditions were minimal suggesting that while carry-over of the storage medium may reduce the aerobic count to a limited extent, it has a minimal effect on the count under ROS-neutralised conditions.

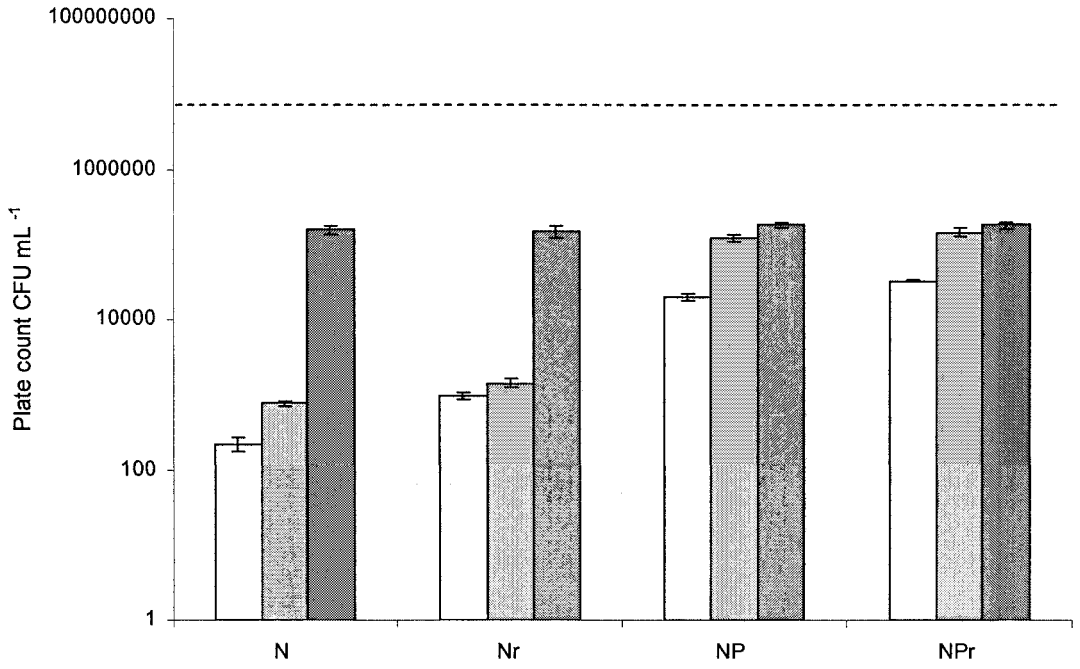


Fig. 3.5 Effects of carry-over of the storage water on enumeration of *Escherichia coli* NCTC8912 from water kept in a brass vessel enumerated on non-selective nutrient agar (N), and nutrient agar plus 0.05% sodium pyruvate (NP), incubated either aerobically (unshaded bars) or in an anaerobic jar (light grey bars), or in an anaerobic cabinet using pre-reduced medium (dark grey bars). Here r in lower case denotes rinsed sample (no-carry-over of stored water). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

3.3.4 Effects of the nutrient status of the enumeration medium on counts of *Escherichia coli* NCTC8912 from water kept in a brass vessel

Plate counts are shown in Figure 3.6 for *E. coli* NCTC8912 after 6 h suspension in water kept in a brass mutka and enumerated on standard nutrient agar, nutrient-rich medium brain-heart agar and the minimal medium R2A in the presence and absence of 0.05% sodium pyruvate either, i) aerobically, ii) anaerobically in an anaerobic jar, or iii) on pre-reduced medium in an anaerobic cabinet. The results indicate that cells cultured aerobically on standard nutrient agar and low-nutrient R2A medium gave a higher count than nutrient-rich medium brain-heart agar for pyruvate supplemented and unsupplemented plates. Counts under anaerobic conditions on unsupplemented growth medium with sodium pyruvate were higher than their aerobic counterparts, for nutrient agar (5-fold) and brain-heart agar (21-fold) whereas they were almost similar on R2A medium. Counts under anaerobic conditions on pyruvate-supplemented growth medium were higher than their aerobic counterparts for all three media, with the greatest relative increase (10-fold) for brain-heart agar. In contrast, no such difference in counts on nutrient agar, brain-heart agar and R2A agar was obtained between pyruvate-supplemented and unsupplemented plates prepared and maintained in the anaerobic cabinet. This resulted in broadly equivalent counts for all three media. Thus smaller differences in counts were obtained between nutrient agar, brain-heart agar, R2A agar under anaerobic conditions and pre-reduced conditions as compared to the aerobic counts.

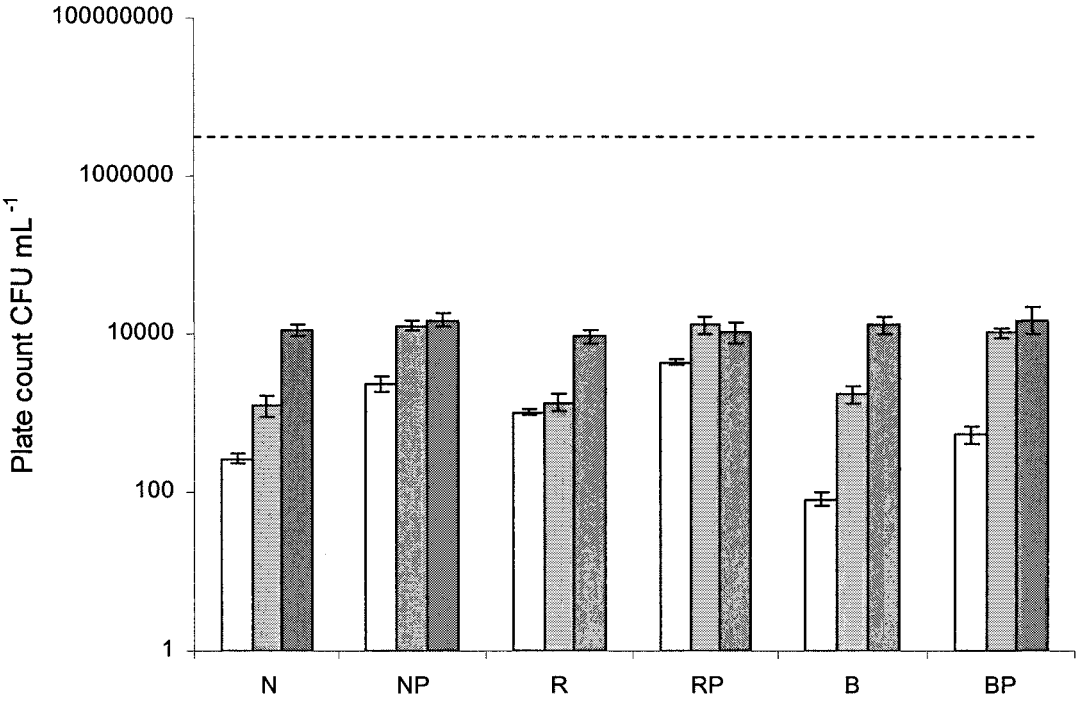


Fig. 3.6 Effects of the nutrient status of the enumeration medium on counts of *Escherichia coli* NCTC8912 from water kept in a brass vessel enumerated on nutrient agar (N), R2A agar (R), and brain-heart agar (B), incubated with and without supplementation of the medium with 0.05% w/v sodium pyruvate (P) either aerobically (unshaded bars) or anaerobically in an anaerobic jar (light grey bars), or anaerobically in an anaerobic cabinet using pre-reduced medium (dark grey bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

3.3.5 Effects of medium composition and selective agents on different strains of *E. coli* from water kept in the brass and earthern storage vessels

Counts are shown in Fig. 3.7a for a suspension of *E. coli* NCTC8912 at 0 h, plated onto either nutrient agar (non-selective medium), MacConkey agar, mFC-R, mEndo agar or mLSA (selective media) and enumerated either (i) aerobically on unsupplemented medium or (ii) under ROS-neutralised conditions (pyruvate-supplemented medium incubated in an anaerobic jar). Similar initial counts were obtained for all media, irrespective of the enumeration conditions. A similar pattern (Fig. 3.7c) was also obtained after 24 h suspension of *E. coli* NCTC8912 in water kept in an earthern vessel with only minimal decreases in counts observed, compared to the initial inoculum.

In contrast, Fig. 3.7b shows *E. coli* NCTC8912 counts following 6 h incubation in water kept in a brass mukta. The overall results showed 20-fold to 20000-fold lower counts when compared to the initial inoculum, with the extent of the decrease showing a strong dependence on both the medium and the growth conditions used. Comparing the selective coliform media incubated under aerobic conditions, MacConkey agar gave a similar count to nutrient agar, mLSA gave the lowest aerobic count and mFC-R together with mEndo medium gave intermediate values. Under ROS-neutralised conditions, nutrient agar showed an increase in colony count of around 20-fold whereas the selective media gave more variable increases, with none of them achieving the ROS-neutralised count observed with nutrient agar. However, the same trend was noted under ROS-neutralised conditions as was observed for aerobic conditions, with MacConkey agar giving a count closest to that of nutrient agar and mLSA giving the lowest overall count.

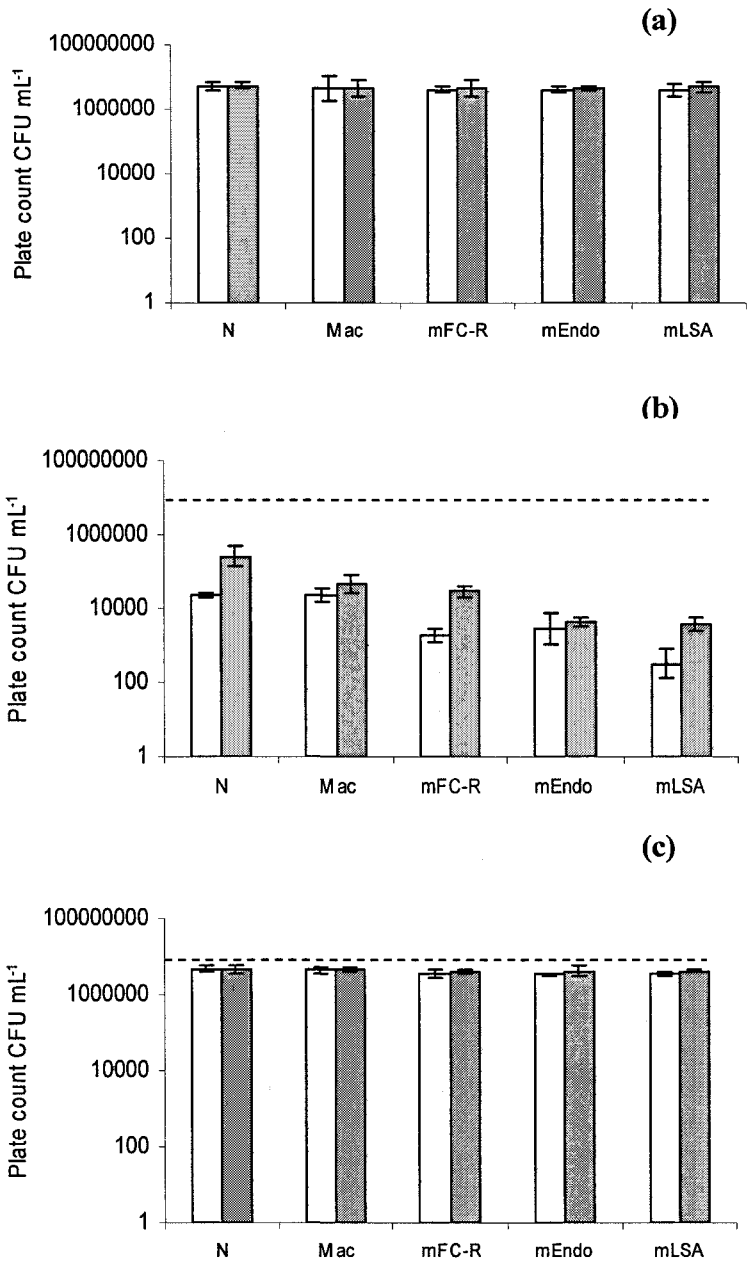


Fig. 3.7 Effects of medium composition and selective agents on *E. coli* NCTC8912 in water stored in the brass and earthern storage vessels (a) initial inoculum at 0 h in sterile distilled water (b) after 6 h in water kept in a brass mutka; (c) after 24 h in water kept in a earthern vessel; enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, and m-FC agar without rosolic acid (mFC-R), incubated either aerobically (unshaded bars) or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (shaded bars; ROS-neutralised conditions). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 3.8 a shows the results of a similar experiment carried out using stationary phase *E. coli* TN675 suspended for 6 h in water kept in a brass mutka, while Fig. 3.8b and c shows equivalent results for the two Indian isolates, *E. coli* PUCC061 and PUCC113, suspended for 24 h in water kept in a brass mutka (after 6 h there was minimal change in the counts of these two environmental isolates). Overall, the broad trends shown in Fig. 3.8 a, b and c are similar to those observed with *E. coli* NCTC8912 (Fig. 3.6b), confirming that non-selective nutrient agar under ROS-neutralised conditions gave substantially higher counts than under aerobic conditions, and compared to all of the selective media, with MacConkey agar proving least inhibitory and mLSA the most inhibitory in all cases. However, a somewhat reduced effect of ROS neutralisation was seen with the two environmental isolates, compared with the laboratory strains, which indicates that they may be somewhat less susceptible to respiratory self-destruction and/or medium-derived peroxides. This is also consistent with their lower overall rate of inactivation in water stored in the brass mutka, requiring 24 h to give broadly similar decreases in plate counts to those observed after 6 h for *E. coli* NCTC8912 (Fig. 3.7b) and TN675 (Fig. 3.8a). However, for all four tested *E. coli* strains, suspensions of cells kept in water for 48 h in the brass mutka gave no detectable counts on nutrient agar or on any selective medium, whether incubated under aerobic or ROS-neutralised conditions, confirming the longer-term inhibitory effects of storage in the brass vessel.

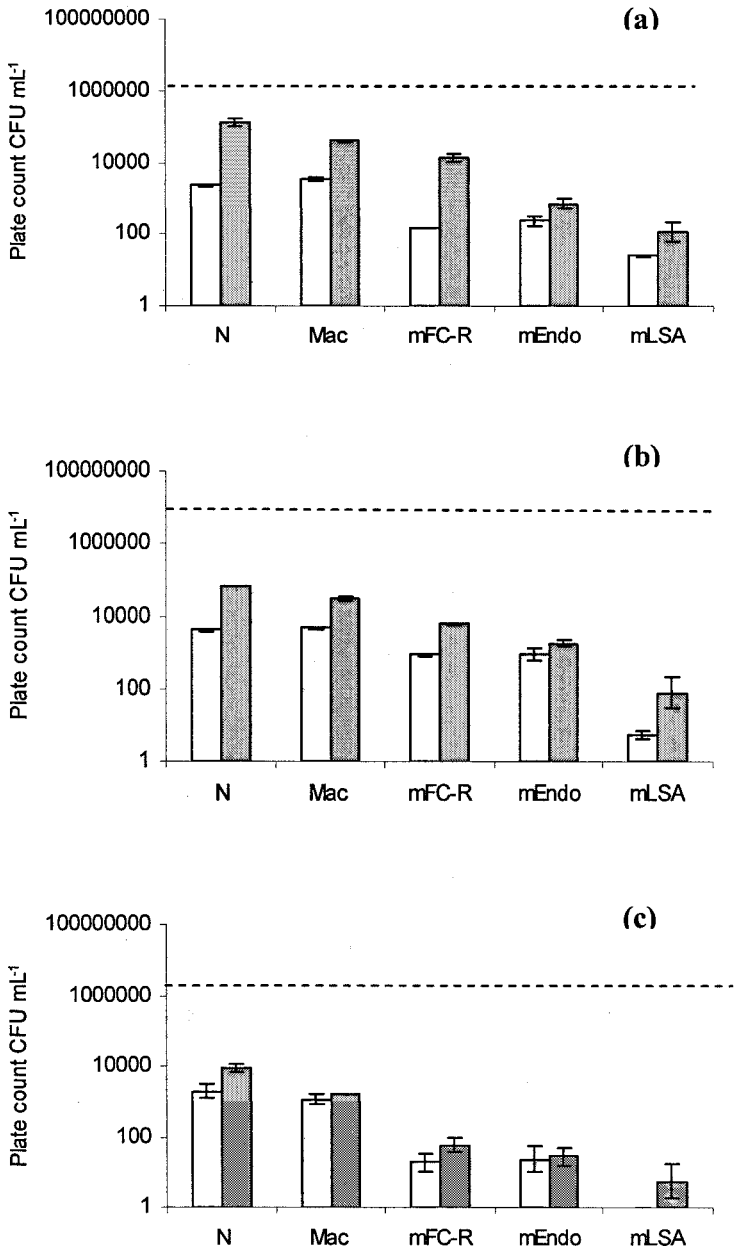


Fig. 3.8 Effects of medium composition and selective agents on the counts of suspensions of three *Escherichia coli* strains in water kept in a brass mutka
(a) *E. coli* TN675 after 6 h storage in water in a brass mutka; (b) *E. coli* PUCC061 after 24 h storage in water in a brass mutka; (c) *E. coli* PUCC113 after 24 h storage in water in a brass mukta, enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, and m-FC agar without rosolic acid (mFC-R), incubated either aerobically (unshaded bars) or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (shaded bars; ROS-neutralised conditions). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

3.2.6 Effects of enumeration conditions on coliform counts for environmental water samples

Environmental water samples: Figures 3.9a, 3.10a and 3.11a respectively show results obtained for presumptive total coliform counts obtained by membrane filtration of three environmental water samples, from Khamanon, Doraha and Khanpur rivers respectively, using four different selective media under aerobic and ROS-neutralised conditions. A broadly similar initial count was obtained for each water sample on all of the selective media used, irrespective of whether the plates were incubated aerobically on unsupplemented medium or anaerobically on pyruvate-supplemented medium, though there was some indication of a slightly higher initial count on MacConkey agar and a slightly lower value on mLSA for Khanpur river water.

Figures 3.9b, 3.10b and 3.11b shows the presumptive total coliform counts obtained when samples of the same three river waters were stored in a brass mutka for 6 h. Overall, the presumptive total coliform (TC) counts decreased by around 100-fold on MacConkey agar, with greater reductions seen for the other three selective media, and especially for mLSA, which gave no detectable count under aerobic conditions in one of the three samples (Fig. 3.8b). As with the pure cultures of *E. coli*, enumeration under ROS-neutralised conditions gave higher counts than conventional aerobic incubation, though the increases observed with the environmental water samples were somewhat less than those seen with the pure cultures (c.f. Fig. 3.1-3.7). After 48 h storage within the brass mutka all three river waters gave no detectable count on any medium, whether incubated under aerobic or ROS-neutralised conditions, confirming the inhibitory effects of brass over a two-day period.

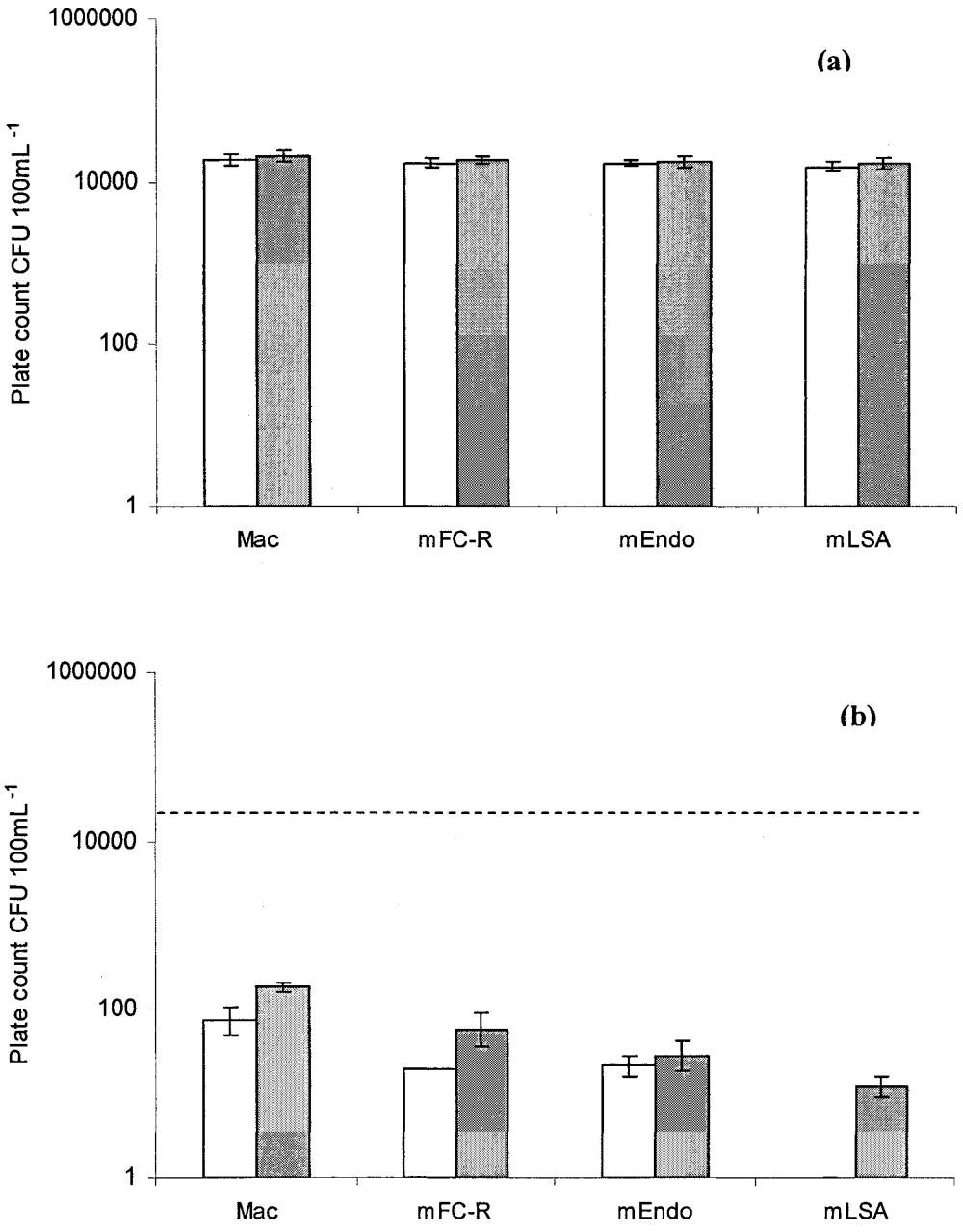


Fig 3.9 Effects of storage on presumptive total coliform (TC) counts of Khamanon river sample (a) at 0 h; (b) after 6h in a brass mutka; enumerated using MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, m-FC agar without rosolic acid (mFC-R), incubated either aerobically (unshaded bars) or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (shaded bars; ROS-neutralised conditions). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

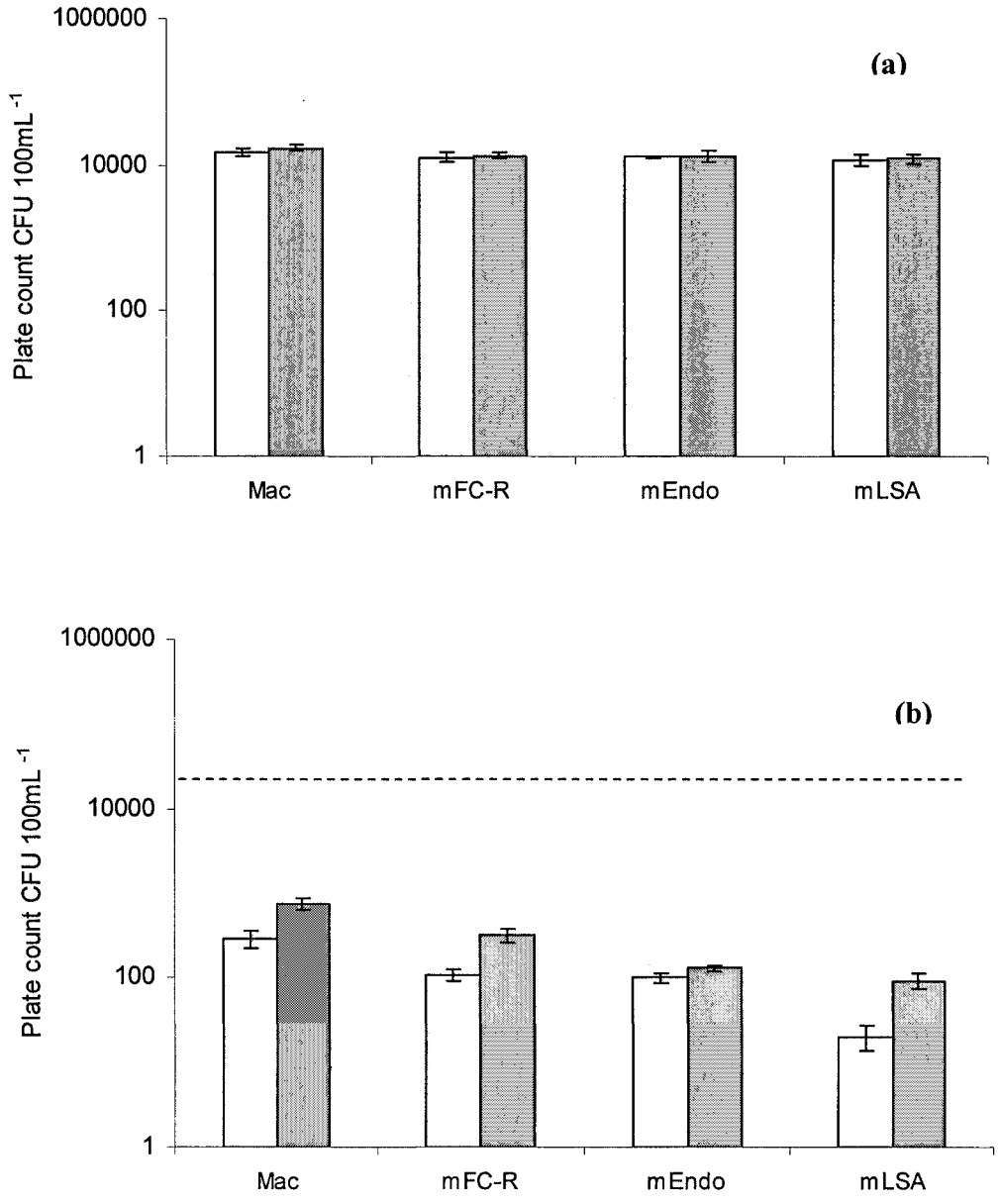


Fig 3.10 Effects of storage on presumptive total coliform counts of Doraha river sample (a) at 0 h; (b) after 6h in a brass mutka; enumerated using MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, m-FC agar without rosolic acid (mFC-R), incubated either aerobically (unshaded bars) or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (shaded bars; ROS-neutralised conditions). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

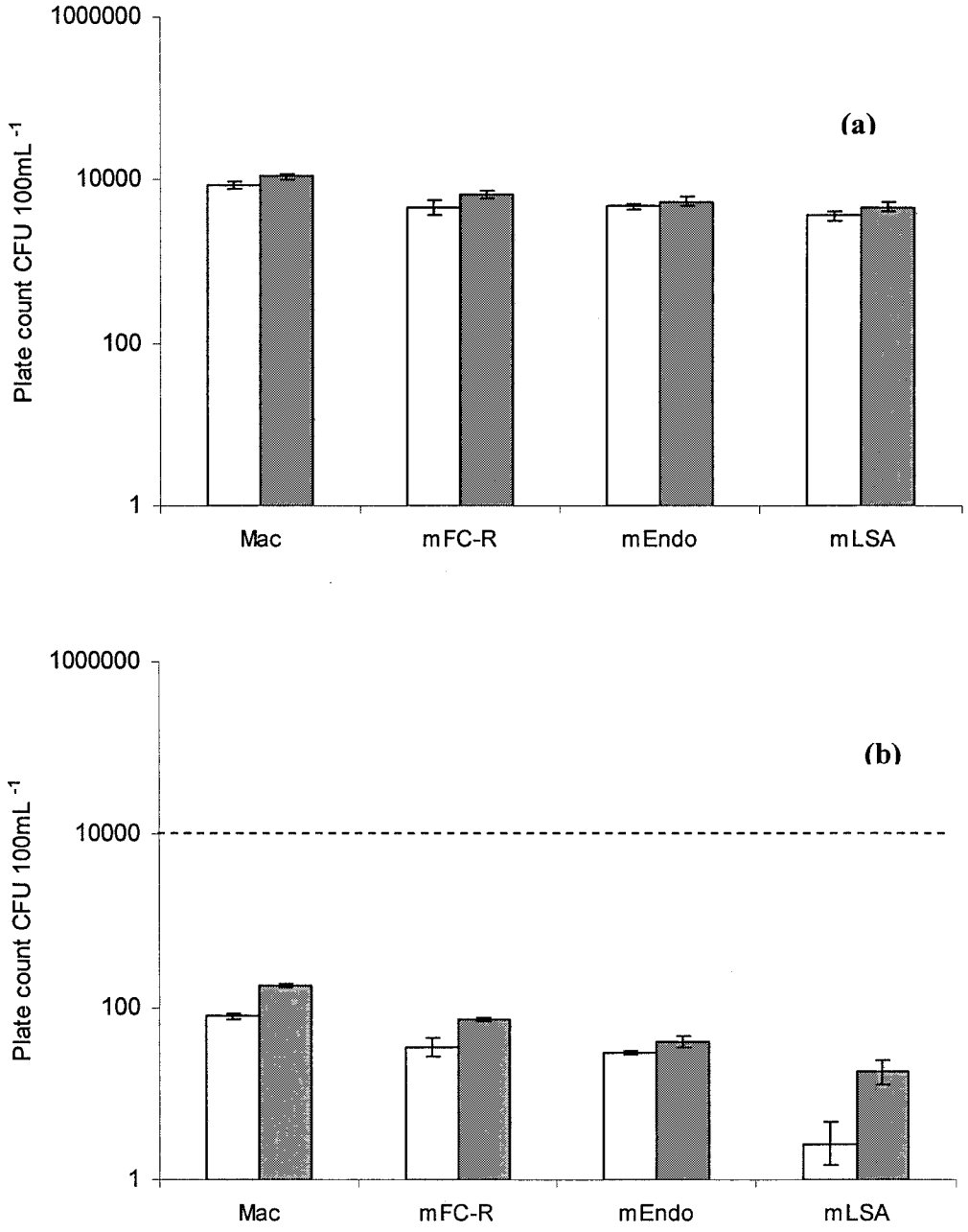


Fig. 3.11 Effects of storage on presumptive total coliform counts of Khanpur river sample (a) at 0 h; (b) after 6h in a brass mutka, enumerated using MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, m-FC agar without rosolic acid (mFC-R), incubated either aerobically (unshaded bars) or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (shaded bars; ROS-neutralised conditions). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Household water samples: Table 3.2 shows data for coliform counts obtained by membrane filtration of water samples collected on twelve occasions from household muktas in the survey villages following overnight storage (12-15 h) alongside counts for the source water used to fill the storage vessels. The mean presumptive total coliform count was obtained for the four tested selective media, both aerobically and under ROS-neutralised conditions, as in previous experiments. The source waters showed some evidence of different counts with the various media used, with the lowest values for mLSA under aerobic conditions. Storage of water overnight in an earthern vessel resulted in a decrease in aerobic count of between 15% and 78%, with MacConkey agar giving the smallest decreases and mLSA the greatest proportional reductions. Under ROS-neutralised conditions, the counts for water stored in the earthern mutkas were somewhat higher, but the same overall trend was observed for the relative performance of the selective media. Overall, the results indicate that while storage overnight in an earthern vessel may cause some sub-lethal damage to a fraction of the cells, there is less evidence of irreversible inactivation, since the ROS-neutralised counts on MacConkey agar were mostly >80% of the corresponding values for the source water.

Table 3.2 also shows the data for brass mutkas, where a far greater effect of overnight storage was observed than was seen with earthern mutkas. The counts obtained differed, depending upon which selective medium was used, in the general sequence (from highest to lowest count): MacConkey > mFC > mEndo > mLSA. The aerobic MacConkey count was typically around 4-8% of that obtained for the source water, with even lower values for the other selective media: for example, in seven of the twelve brass mutka data sets listed in Table 3.2, no colony count was obtained for triplicate sample of 100 mL of water enumerated aerobically on mLSA.

Table 3.2 Presumptive total coliform (TC) counts (CFU per 100 Ml) for source and overnight stored water from households in three rural locations in Panjab Mean counts (upper and lower 95% confidence limits shown in brackets below each mean value) for presumptive total coliforms enumerated from each water sample using a range of selective media, incubated either aerobically using unsupplemented medium or under ROS-neutralised conditions i.e. anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar . S: source water (stored overnight in refrigerator). E: water from earthern mutka (12-15 h storage, ambient temperature). B: water from brass mutka (12-15 h storage, ambient temperature). Samples noted as <1, gave no detectable coliforms in triplicate 100 ml samples.

SOURCE Date of sampling	Sample Type	MacConkey agar aerobic	MacConkey agar ROS-neutralised	m-Faecal Coliform agar-rosolic acid aerobic	m-Faecal Coliform agar-rosolic acid ROS-neutralised	m-Endo agar aerobic	m-Endo agar ROS-neutralised	m-lauryl sulphate agar aerobic	m-lauryl sulphate agar ROS-neutralised
1. CHANDIGARH	S	7032	7564	6230	6766	6196	6665	5630	6033
20.2.03		(6407-7718)	(6552-8731)	(5391-7201)	(6289-7278)	(5166-7615)	(5832-7615)	(4797-6609)	(5791-6286)
	E	5933	6766	2932	3864	2461	3665	1830	2330
		(5690-6185)	(6137-7457)	(2470-3479)	(3257-4589)	(1722-3517)	(3205-4192)	(1283-2607)	(1763-3080)
	B	97.6	183.3	35	57.3	15.7	20.6	<1	7.3
		(82-117)	(160-210)	(26.8-44.7)	(52.5-62.5)	(11.5-21)	(16.3-26)		(5.3-10.2)
2. DORAHA	S	6929	7497	6432	6766	6129	6399	3397	4862
21.2.03		(5725-8386)	(6433-8737)	(5810-7121)	(6137-7458)	(5142-7308)	(5984-6844)	(2719-4243)	(3888-6081)
	E	5899	6696	3728	4660	2363	2864	732	1766
		(5485-6346)	(5522-8118)	(2794-4974)	(3525-6160)	(1785-3129)	(2273-3609)	(525-1020)	(1532-2035)
	B	383	590	275	343	256	316	20.3	123
		(302-485)	(485-717)	(164-462)	(284-415)	(167-392)	(257-390)	(14.7-28)	(73-207)
3. DORAHA	S	3766	5598	2766	3533	2666	3333	2230	2564
26.2.03		(3304-4291)	(4800-6537)	(2528-3028)	(3294-3769)	(2428-2928)	(3095-3589)	(1666-2984)	(1979-3320)
	E	3227	5092	2365	2933	2163	2866	1662	2130
		(2313-4501)	(3770-6876)	(1923-2909)	(2696-3190)	(1591-2440)	2627-3127()	(1113-2482)	1570-2889()
	B	66.7	167.7	30	73.7	20.6	46.6	<1	13.2
		(58.3-76.16)	(189-177)	(23.5-38.3)	(67-80.5)	(11.5-36.7)	(38.6-56)		(6.6-38.3)
4. GUJRAWALA	S	1417	1773	950	1146	816	973	723	887
27.2.03		(1352-1484)	(1668-1885)	(843-1070)	(967-1358)	(660-1008)	(845-1120)	(623-839)	(823-955)
	E	1270	1677	886.3	1003	666.2	860	486	637
		(1187-1359)	(1612-1744)	(757-1037)	(979-1028)	(548-810)	(778-950)	(364-649)	(589-688)
	B	52.7	131	27.7	66	24.3	35.6	12	24.6
		(37.9-73)	(120-142)	(25-30)	(51-86)	(19.8-30)	(25-51)	(4.75-29.4)	(16-38.3)
5. DORAHA	S	8098	9796	7296	8297	6896	7966	5461	6699
25.3.03		(7282-9006)	(8427-11387)	(6113-8707)	(7242-9507)	(5719-8314)	(7487-8476)	(4302-6932)	(5999-7480)
	E	5732	7832	3965	4621	2854	3700	1926	2723
		(5114-6425)	(7204-8515)	(3356-4684)	(3160-6758)	(1734-4696)	(3309-4291)	(1197-3099)	(1665-4448)
	B	386	1079	180	357	93	255	<1	63
		(268-556)	(872-1336)	(180)	(311-409)	(53-162)	(130-498)		(43-93)

SOURCE Date of sampling	Sample Type	MacConkey agar aerobic	MacConkey agar ROS-neutralised	m-Faecal Coliform agar-rosolic acid aerobic	m-Faecal Coliform agar-rosolic acid ROS-neutralised	m-Endo agar aerobic	m-Endo agar ROS-neutralised	m-lauryl sulphate agar aerobic	m-lauryl sulphate agar ROS-neutralised
6. GUJRAWALA	S	9830	11996	6996	8566	5561	6925	4098	4932
25.3.03		(8655-11166)	(10774-13359)	(5818-8412)	(8086-9075)	(4400-7029)	(5379-8917)	(3424-4903)	(4457-6841)
	E	8129	10428	5498	6629	3593	4729	2160	3227
		(6870-9619)	(8820-12329)	(4701-6428)	(5429-8093)	(2603-4960)	(3766-5938)	(1440-3241)	(2313-4501)
	B	706	1343	292	633	73.2	200	<1	4.7
		(546-913)	(1179-1529)	(185-462)	(534-751)	(52.5-102)	(200)		(2.7-8.7)
7. DORAHA	S	6998	8966	6398	7098	5931	6198	4230	5596
3.4.03		(6189-7913)	(8331-9649)	(5593-7318)	(6288-8012)	(5094-6905)	(5395-7120)	(3415-5238)	(4577-6841)
	E	4560	6727	2154	3863	2296	3066	1297	1898
		(3429-6064)	(5379-8413)	(1238-3749)	(3072-4857)	(1671-3153)	(2827-3326)	(931-1807)	(1513-2381)
	B	37.7	57.3	13.3	30.6	12.6	22.3	1.59	11
		(31.6-44.9)	(47.5-69)	(11.1-16)	(24.7-38)	(8.6-18.5)	(16.7-30)	(0.28-8.87)	(6.8-16.7)
8. GUJRAWALA	S	7197	8132	6998	7896	7098	7466	5294	6798
9.4.03		(6153-8417)	(7503-8814)	(6189-7913)	(6706-9298)	(6396-7878)	(6835-8155)	(4149-6755)	(5990-7715)
	E	3729	5298	1498	2759	1662	2065	500	1066
		(2925-4755)	(4609-6090)	(1124-1996)	(1857-4099)	(1113-2482)	(1630-2616)	(500)	(841-1350)
	B	49.2	130	26.3	36	19.3	25.3	<1	7.3
		(33-72.8)	(123-136)	(17.4-40)	(29-44)	(17-22)	(20-33)		(5.25-10.2)
9. CHANDIGARH	S	693	723	626	653	513	590	483	526
9.4.03		(645-745)	(623-839)	(527-745)	(590-722)	(452-583)	(548-635)	(400-582)	(428-645)
	E	373	530	150	275	165	206	50	123
		(293-475)	(461-609)	(85-266)	(186-410)	(111-248)	(163-262)	(50)	(73-207)
	B	48	70	22	37	17	25	<1	7
		(35-67)	(58-84)	(14-32)	(29-44)	(15-20)	(20-33)		(4-13)
10. CHANDIGARH	S	850	910	606	716	546	663	506	616
29.4.03		(768-940)	(828-1000)	(524-702)	(597-859)	(484-617)	(563-780)	(425-603)	(500-761)
	E	603	713	300	410	317	326	247	283
		(541-672)	(650-781)	(227-361)	(410)	(271-370)	(223-456)	(223-273)	(225-356)
	B	40	96	20	34	23	25	2	7.3
		(32-50)	(79-109)	(16-25)	(26-40)	(20-25)	(25)	(0.3-8)	(5-)
11. DORAHA	S	8298	9062	6998	8600	6862	7098	6295	6798
29.4.03		(7481-9204)	(7667-10710)	(6189-7913)	(8180-9041)	(5674-8299)	(6288-8012)	(5129-7727)	(5990-7715)
	E	6032	7367	5433	5832	3165	3496	2000	2664
		(5412-6723)	(6189-7913)	(4955-5956)	(5213-6524)	(2710-3697)	(2734-4471)	(2000)	(2077-3416)
	B	42	79.7	20	33.7	22.7	25	<1	6.7
		(34-51.5)	(73-86.5)	(16-25)	(29-39)	(20.3-25.3)	(25)		(4.53-9.75)
12. CHANDIGARH	S	14231	17298	12999	15899	12898	13600	9933	11999
15.5.03		(12972-15612)	(16055-18638)	(12167-13888)	(15062-16783)	(11670-14254)	(13176-14063)	(9447-10444)	(11169-12890)
	E	9798	12566	6360	8099	6400	6964	4932	5767
		(8717-11012)	(12081-13071)	(5011-8072)	(7391-8874)	(5984-6844)	(6030-8042)	(4457-5459)	(5523-6021)
	B	303	760	33.3	76.7	33.3	55.3	18.3	28.3
		(279.6-329)	(689-837.6)	(24-46)	(70-83.5)	(22.26-50)	(53-58)	(16-21)	(23.7-33.8)

The ROS-neutralised counts for water from the brass mutkas were often around two or three times higher than those of the corresponding aerobic counts (Table 3.2). The increases in ROS-neutralised counts depended upon the selective medium used, with MacConkey agar generally showing the highest increase and mEndo agar showing the least increase in count.

Taken together with the results for the pure cultures and the environmental water samples, the results in Table 3.2 indicate that storage in a brass vessel can cause both (i) sub-lethal damage, as demonstrated by the reduced counts under aerobic conditions and on more inhibitory selective media such as mLSA, and (ii) irrevesible inactivation, as evidenced by the substantial decrease in the counts of stored water obtained under ROS-neutralised conditions on less inhibitory medium such as MacConkey agar.

3.3.7 Effects of ROS-neutralisation on enumeration of *Enterococcus faecalis* NCTC775 from water kept in brass and earthern storage vessels

Figure 3.12a shows plate counts for *E. faecalis* NCTC775 after 12 h storage in water kept in a brass mutka (preliminary studies for water stored for 6 h suspension in a brass mutka gave a minimal change in count) while 3.12b shows counts for water stored for 12 h in an earthern vessel, enumerated on nutrient agar with and without 0.05% w/v sodium pyruvate incubated either under standard aerobic conditions, or under one of three different sets of anaerobic conditions i.e. either in (i) an anaerobic jar, or (ii) an anaerobic cabinet or, (iii) an anaerobic cabinet using pre-reduced medium (kept for 24 h in the anaerobic cabinet prior to use). This experiment is equivalent to that shown in Figs 3.1 and 3.2 for *E. coli* NCTC8912. Plate counts for brass-stored *E. faecalis* showed substantial decreases in count as compared to the initial inoculum, with a

reduction of between 10-fold to 120-fold, depending upon the enumeration conditions. Enumeration under aerobic conditions using unsupplemented medium showed the lowest count, with pyruvate-supplemented plates giving a 3-fold higher count than for unsupplemented medium. A further enhancement in count was seen for pyruvate-supplemented nutrient agar plates prepared in air and than transferred either to the anaerobic jar or to the anaerobic cabinet when compared with unsupplemented medium. However, no such difference in counts was obtained between pyruvate-supplemented and unsupplemented plates which were pre-reduced in an anaerobic cabinet prior to use. The plate counts obtained for pyruvate-supplemented media incubated in an anaerobic jar or an anaerobic cabinet were equivalent to those obtained when using pre-reduced medium in an anaerobic cabinet. These results demonstrate that in any of the above enumeration conditions, the damaging effects of ROS on *E. faecalis* can be neutralised. Overall, the enhancement in counts observed under ROS-neutralised conditions was smaller than that seen for *E. coli* NCTC8912 (c.f. Fig. 3.1b), which may be linked to the lack of respiratory metabolism in *E. faecalis*.

In Figure 3.12b for water kept in the earthern vessel for 12 h the results clearly show no substantial decrease compared to the initial inoculum. Thus the enumeration of bacteria on nutrient agar with or without the inclusion of sodium pyruvate, under standard aerobic conditions or under any of the three anaerobic conditions tested gave no discernible difference in count. In this respect, the results obtained for *E. faecalis* NCTC775 are similar to those obtained for *E. coli* NCTC8912 stored in an earthern vessel, shown in Fig. 3.2a-b.

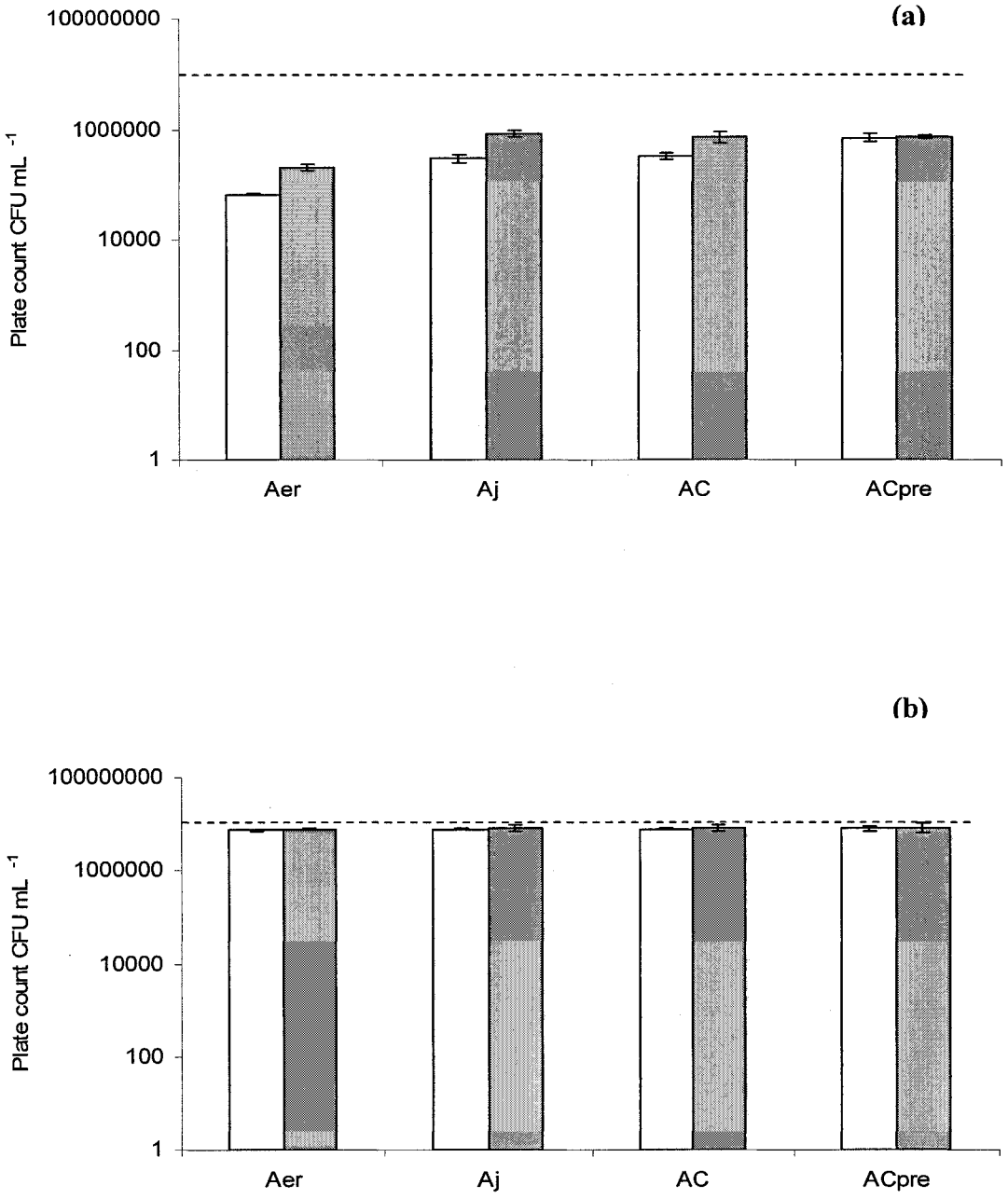


Fig 3.12 Effects of ROS-neutralisation on the enumeration of *Enterococcus faecalis* NCTC775 in water kept in brass and earthern storage vessels (a) 12 h storage in a brass mutka, (a) 12 h storage in an earthern vessels, enumerated on nutrient agar (unshaded bars) or on nutrient agar supplemented with 0.05% w/v sodium pyruvate (shaded bars) prepared and cultured under aerobic conditions (Aer), prepared under aerobic condition and cultured either in an anaerobic jar (Aj) or in an anaerobic cabinet (AC), or using pre-reduced medium maintained in an anaerobic cabinet (ACpre). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

3.3.8 Effects of the nutrient status of the enumeration medium on counts of *Enterococcus faecalis* NCTC775 from water kept in a brass vessel

Figure 3.13 shows the plate counts for *E. faecalis* NCTC775 after 12 h suspension in water kept in a brass mutka and then enumerated using standard nutrient agar, brain-heart agar and the minimal medium R2A in the presence and absence of 0.05% sodium pyruvate incubated at 37^0C under either (i) aerobic conditions (unsupplemented medium), or ii) ROS neutralised conditions (pyruvate-supplemented medium incubated in an anaerobic jar). In contrast to *E. coli* NCTC8912 (Fig. 3.6), the results indicate that brass-stored *E. faecalis* cells cultured aerobically on standard nutrient agar and brain heart agar gave a higher count than nutrient-deficient R2A for pyruvate supplemented and unsupplemented plates. Counts under anaerobic conditions on growth medium unsupplemented with sodium pyruvate were higher than their aerobic counterparts, for nutrient agar (3-fold), brain heart (2-fold) and R2A (12-fold). A further slight enhancement in count was seen for all three media under anaerobic conditions on growth medium supplemented with sodium pyruvate compared with the unsupplemented medium. The nutrient-deficient medium, R2A showed a greater increase in count under anaerobic conditions with and without pyruvate when compared with the aerobic counterparts, whereas both nutrient agar and brain heart agar showed smaller differences. Under ROS-neutralised conditions the nutrient-rich and low nutrient media all gave a broadly similar count.

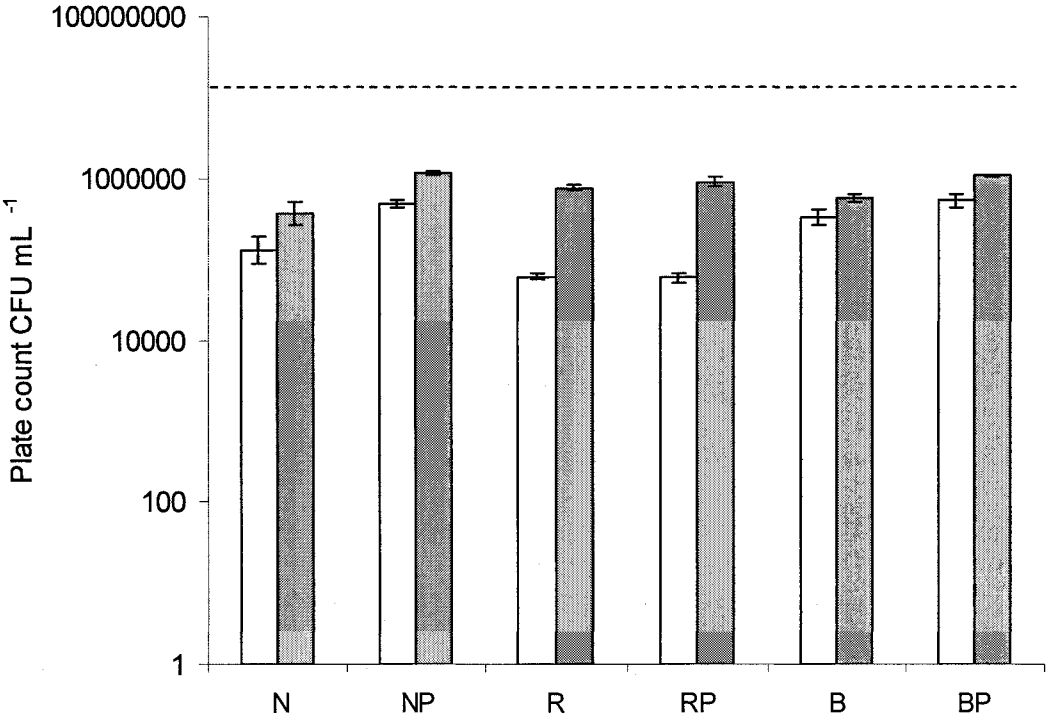


Fig. 3.13 Effects of the nutrient status of the enumeration medium on counts of *Enterococcus faecalis* NCTC775 from water kept in a brass vessel enumerated on nutrient agar (N), R_2A agar (R), and Brain heart agar (B), incubated with and without supplementation of the medium with 0.05% w/v sodium pyruvate (P) either aerobically (unshaded bars) or in an anaerobic jar (dark grey bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

3.3.9 Effects of medium composition and selective agents on different strains of *Enterococcus faecalis* from water kept in a brass vessel

Counts are shown in Fig. 3.14 for a suspension of *E. faecalis* NCTC775 kept in a brass mutka for 12 h. Enumeration was performed using nutrient agar (non-selective medium), and various selective media, incubated either (i) aerobically with unsupplemented medium, or (ii) under ROS-neutralised conditions (anaerobically with supplementation of 0.05% w/v sodium pyruvate in the medium). The overall results showed 35-fold to 1130-fold lower counts when compared to the initial inoculum in all cases, with the extent of the decrease showing a strong dependence on both the medium and the growth conditions used. Comparing the unsupplemented selective media incubated under aerobic conditions, bile aesculin agar gave a count closest to nutrient agar, whereas KF streptococcus agar gave the lowest aerobic count and the other two media gave intermediate values. Comparing the results obtained under ROS-neutralised conditions, KF streptococcus agar showed the greatest increase in count, giving a value that was almost the same as nutrient agar. Slanetz and Bartley agar and bile aesculin agar gave intermediate values whereas MacConkey agar number 2 gave the lowest count under ROS-neutralised conditions, showing a minimal increase from its aerobic counterpart. Non- selective nutrient agar under ROS-neutralised conditions showed an increase in count of around 3-fold compared to aerobic unsupplemented medium, whereas the selective media gave more variable increases i.e. KF streptococcus agar (24-fold), bile aesculin agar (2-fold), Slanetz and Bartley agar (3-fold) and MacConkey agar (2-fold)

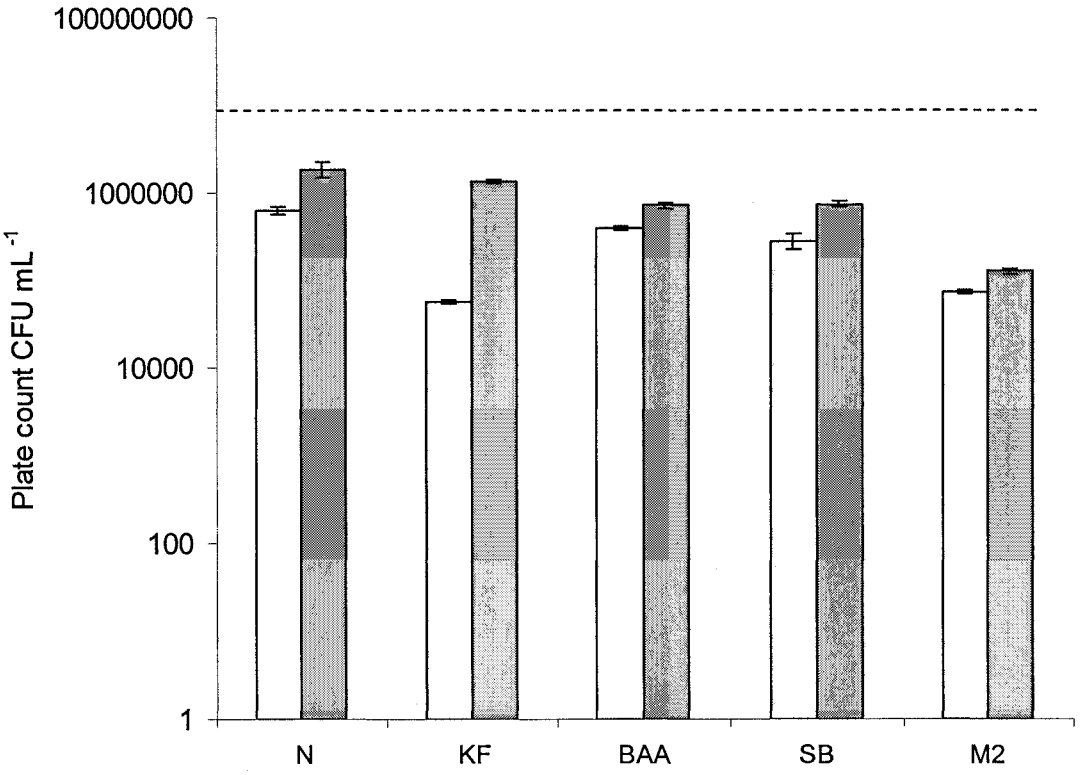


Fig. 3.14 Effects of medium composition and selective agents on *Enterococcus faecalis* NCTC775 from water kept in a brass vessel following 12 h storage, enumerated on non-selective nutrient agar (N), KF streptococcus agar (KF), bile aesculin agar (BAA), Slanetz and Bartley agar (SB) or MacConkey agar number 2 (M2), incubated either aerobically in unsupplemented medium (unshaded bars) or under ROS-neutralised conditions i.e. medium supplemented with 0.05% sodium pyruvate and incubated in an anaerobic jar (shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 3.15a shows a similar set of experiments carried out using *E. faecalis* ATCC35550 suspended for 12 h in water stored in the brass mutka, while Fig. 3.15b and c shows equivalent results for the two Indian isolates, *E. faecalis* PTO1 and PTO2, suspended for 24 h in the brass mutka (as 12 h incubation in the brass mutka was not sufficient to substantially affect the count of these two environmental isolates), enumerated using a narrower range of media than for *E. faecalis* NCTC775 which consisted of nutrient agar, KF streptococcus agar and Slanetz and Bartley agar either (i) aerobically in standard unsupplemented medium, or (ii) under ROS-neutralised conditions (pyruvate-supplemented medium incubated in an anaerobic jar). The results obtained in Fig. 3.15a, b and c are similar to those observed with *E. faecalis* NCTC775 (Fig. 3.14) with all 3 strains showing enhanced counts (of 2-fold to 5-fold) under ROS-neutralised conditions when compared to the aerobic counterparts.

All four of the tested strains of *E. faecalis* suspensions gave no detectable counts on nutrient agar or on any selective medium after 48 h suspension in water kept in the brass mutka whether incubated under aerobic or ROS-neutralised conditions, in agreement with the inhibitory effects of brass incubation noted for all strains of *E. coli* and coliforms after 2 days, as mentioned earlier. Furthermore, all four strains of *E. faecalis* showed a reduced effect of ROS neutralization in selective media when compared to *E. coli* (e.g. Fig. 3.7 and Fig. 3.8).

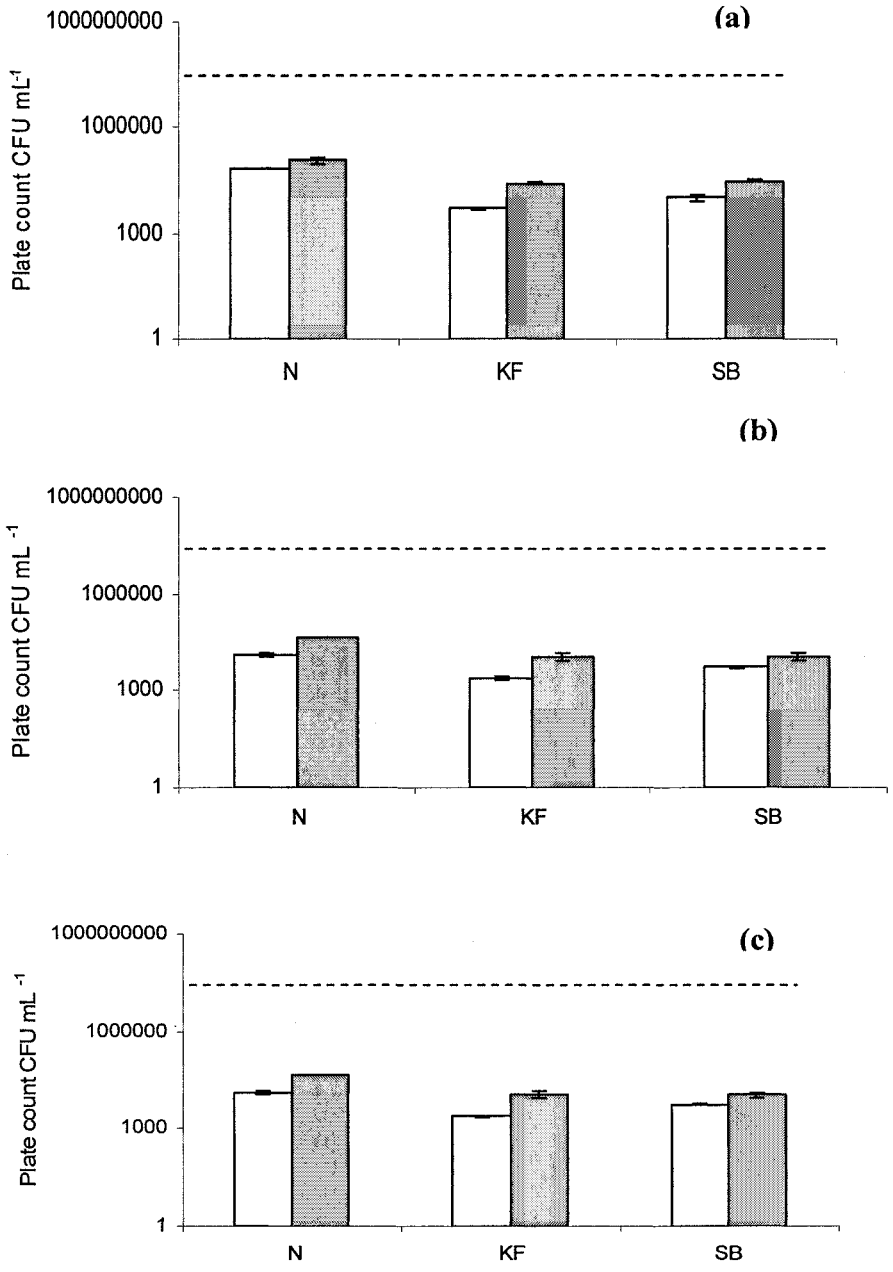


Fig. 3.15 Effects of medium composition and selective agents on different strains of *Enterococcus faecalis* from water kept in a brass vessel (a) *E. faecalis* ATCC35550 kept for 12 h (b) *E. faecalis* PTO1 kept for 24 h, (c) *E. faecalis* PTO2 kept for 24 h, enumerated on non-selective nutrient agar (N), KF streptococcus agar (KF), or Slanetz and Bartley agar (SB), incubated either aerobically in unsupplemented medium (unshaded bars) or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

3.4 Discussion

The inhibitory effects of heavy metals such as copper, zinc and silver on aquatic micro-organisms have been the subject of scientific study for over a century (Jonas, 1989). Their biocidal properties have been exploited for control purposes, e.g. copper has been used to inhibit heterotrophic microbes (Lin *et al.,* 1998), and veterinary pathogens (Beal *et al.*, 2004) and to counter microbial biofilms in antifouling preparations (Kielemoes and Verstraete, 2001). The antimicrobial effects of silver have been rediscovered more recently (Pedazhur *et al.,* 1997), with the development of medical applications including silver-impregnated catheters (e.g. Samuel and Guggenbichler, 2004), dressings for wound care (Percival *et al.,* 2005), per-nasal drug administration devices (Klocker *et al.,* 2004) and water disinfection systems (Butkus *et al.,* 2004; Pedazhur *et al.*, 1997). Commercial products such as EEKO-BALL (containing silver and copper at low levels) have been used to control bacterial growth in cooling towers (Kim *et al.,* 2004). The toxic effects of these heavy metals are thought to result from the solubilisation of small amounts of metal ions from surfaces, e.g. from metal pipes and vessels, and the term 'oligodynamic effect', was coined in 1893 to emphasise that this phenomenon occurs at extremely low metal ion concentrations. However, the influence of oxygen status on the enumeration of these metal-stressed microbes does not appear to have been considered in detail in earlier studies.

In the present study the levels of copper ions present in water stored in a brass mutka for several days were within the maximum levels recommended for human consumption of this metal (Hotz *et al.*, 2003). The recommended daily dietary intake for copper for an adult is 2 mg per day (WHO, 2004a). Even after consuming several

litres of water stored for 48 h in a brass mutka in a single day, a person would be well within the daily recommended intake for both metals from this source (Domek *et al.*, 1984; Fitzgerald, 1998; Milne, 1998; Grey and Steck, 2001). Thus drinking of water stored in a brass mutka should not lead to any major additional risk to human health, since the measured levels of copper remained were approximately 0.15 mg L^{-1} after 48 h storage (Fig. 3.3). Some concerns have been expressed over the occurrence of Indian childhood cirrhosis (ICC) as a result of the use of brass vessels in rural households (Zietz *et al.*, 2003; Magdalena *et al.*, 2003). However, the incidence of ICC has been reported to be low in rural families storing water in brass vessels and high only when families stored and boiled milk in brass vessels (Tanner, 1998).

The toxicity of oxygen to bacteria growing under conventional aerobic conditions is due primarily to partially reduced forms of oxygen produced (i) due to oxidation of nutrients during autoclaving and (ii) during respiration (Stephens *et al.*, 2000). The reduced by-products of oxygen produced during respiration include the superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2; Eqn. 3.1) and the highly reactive hydroxyl radical ($OH^{\bullet}$): all of these are commonly referred to as reactive oxygen species (ROS). Most of these compounds are generated from sequential univalent reductions of molecular oxygen catalyzed by several membrane-associated respiratory enzymes (Cabiscol *et al.*, 2000; Droge, 2002). Hydrogen peroxide, a secondary form of reactive oxygen, is also produced by enzymatic systems in white blood cells for the destruction of bacteria by phagocytosis (Farr and Kogoma, 1991; Brock *et al.*, 1997). It can be generated whenever two $O_2^{-\bullet}$ radicals and two protons combine in the presence of enzyme superoxide dismutase by the following reaction:

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(Eqn. 3.1)}$$

The hydrogen peroxide will be present whenever the superoxide anion radical ($O_2^{-\bullet}$) is formed within a bacterial cell. Thus most of the damage caused by the generation of reactive oxygen species may be attributed to the production of H_2O_2 since it is a long-lived end product, unlike some of the other unstable intermediates (Droge, 2002; Khaengraeng, 2004). H_2O_2 in turn is known to produce other free radicals such as $OH^{\bullet}$ by a number of ways of which one, the Fenton reaction, is shown below. In this reaction the superoxide radical $O_2^{-\bullet}$ reduces Fe^{3+} to Fe^{2+}, which then reacts with H_2O_2 to produce hydroxyl anions, hydroxyl radicals and regenerate Fe^{3+} (Juven and Pierson, 1996). The hydroxyl radical is capable of oxidising almost any cellular component including proteins, nucleic acids and lipids (Stephens *et al.*, 2000). Thus the superoxide radical can lead to the formation of the most reactive and severely toxic free radical i.e. the hydroxyl radical, mediated through the metal-catalysed Fenton reaction (Duesterberg *et al.*, 2005), since intracellular oxidases in the presence of transition metal ions such as iron or copper produce free radicals according to the following two equations:

$$O_2^{-\bullet} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad \text{(Eqn. 3.2)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \qquad \text{(Eqn. 3.3)}$$

In response to the production of reactive oxygen species, cells maintain a variety of defences against oxygen toxicity. There are many enzymes that have evolved to deal with oxidative stress, including superoxide dismutase (SOD) and catalase (Loewen, 1996). Prokaryotes with respiratory metabolic pathways such as *E. coli* contain both of these enzymes which are generated during oxidative stress e.g. when redox-cycling

compounds enter cells and generate ROS, they can induce an increase in the activity of such enzymes (Farr and Kogoma, 1991; Simpson *et al.*, 1992).

In contrast, *E. faecalis* is an aerotolerant anaerobe, which can survive and grow in the presence of oxygen but which metabolizes carbohydrates by employing fermentation and oxygen toxicity is countered by an alternative method (Flahaut *et al.*, 1998). *E. faecalis* does not possess catalase but it synthesizes flavin-dependent oxidase and peroxidase enzymes on exposure to molecular oxygen or H_2O_2 (Crane *et al.*, 1995; Flauhat *et al.*, 1996; Rince *et al.*, 2000).

Several researchers have studied the influence of oxygen and its reactive by-products (ROS) on bacterial enumeration following exposure to various external stressors which have been discussed in detail in Chapter 4. Thus, enhanced counts of *Escherichia coli* after freezing or heat injury have been observed when compounds such as catalase or pyruvate, both of which neutralise peroxides by causing their conversion to less reactive by-products, have been added to non-selective or selective growth media (e.g. Lee and Hartman, 1989; Mackey and Derrick, 1986; Mackey and Seymour, 1987; Czechowicz *et al.*, 1996). Bogosian *et al.* (2000) explained the decomposition of peroxides which are produced from constituents in the growth medium during autoclaving as the reason for the increase in the aerobic count of cold-stored *Vibrio vulnificus* after incorporation of peroxide scavengers into enumeration media. They noted a 1000-fold higher count of cold-stored cells on aerobic media containing scavengers of peroxides compared with media without such constituents. Similar enhancements in counts were observed by Mizunoe *et al.* (1999) when sodium pyruvate or catalase was incorporated in media used to enumerate temperature-stressed

E. coli O157. The mode of action of sodium pyruvate is to react spontaneously with hydrogen peroxide to produce acetic acid, water and carbon dioxide (Khaengraeng, 2004), according to the following equation:

$$H_2O_2 + CH_3COCOOH \rightarrow CH_3COOH + H_2O + CO_2 \qquad \text{(Eqn. 3.4)}$$

Similarly catalase reacts with two molecules of with hydrogen peroxide to produce water and oxygen molecules according to the following equation:

$$2H_2O_2 + O_2 \rightarrow 2H_2O + O_2 \qquad \text{(Eqn. 3.5)}$$

The work of Stevens *et al.* (2000) points to two sources of oxidative stress during culture, namely from ROS due to (i) reactive components of the growth medium and (ii) cellular respiration, the latter being responsible for the phenomenon of respiratory self-destruction of sub-lethally damaged cells under aerobic conditions (Dodd *et al.*, 1997; Aldsworth *et al.,* 1999). In contrast, under anaerobic conditions, facultative bacteria such *as E. coli* will satisfy their carbon and energy requirements using fermentative pathways, rather than aerobic respiration, thereby avoiding respiratory self-destruction. The fact that heavy metals such as copper are know to generate hydroperoxide free radicals (Rodriguez-Montelongo *et al.*, 1993) and to damage cellular catalase (Calabrese and Bissonnette, 1990b) provides a plausible explanation of the effects observed in the present study, since damage to the catalase would then render cells growing under aerobic conditions sensitive to their own respiratory by-products, as a side-effect of such toxicity. Thus, despite the fact that respiratory metabolism itself may be substantially reduced in metal-stressed *E. coli* (Domek *et al.*, 1984; 1987), it would seem most likely that cellular antioxidant defence systems may

show proportionately greater damage, resulting in the toxicity observed under aerobic conditions. In contrast, since *E. faecalis* is an aerotolerant anaerobe without any respiratory metabolism, the reduced count observed under aerobic conditions must be due to non-respiratory ROS, such as medium-derived peroxides produced due to auto-oxidation of medium components, which may account, in part, for the reduced ROS-sensitivity of *E. faecalis* compared to *E. coli*.

A beneficial effect of adding pyruvate was observed under aerobic conditions in the present study for the enumeration of *E. coli* and, to a lesser extent, *E. faecalis* kept in water stored in a brass vessel (Figs. 3.1-3.10; 3.11-3.14). Enterococci are more resistant indicator bacteria than *E. coli* (Anon., 2002), therefore showing less sub-lethal damage. However, a further enhancement in counts was obtained on transfer of cells plated onto pyruvate-supplemented medium to anaerobic conditions (anaerobic jar or anaerobic cabinet). This indicates that while peroxides may be quantitatively the most important ROS, they are not the sole source of the growth inhibition observed under standard aerobic conditions, and that additional sources of ROS must be considered and eliminated. Consequently, the addition of sodium pyruvate is only partially successful in counteracting the effects of ROS on agar-based media under aerobic conditions.

It is possible to interpret the results of the present study for *E. coli* in terms of the hypothesis of respiratory self-destruction (Aldsworth *et al.*, 1999). A diagrammatic representation of the relative proportion of healthy, injured and inactivated bacterial cells after brass-injury is shown in Fig. 3.16. The aerobic count of bacteria kept in water stored in the brass vessel and then enumerated on a non-selective medium (nutrient agar) represents the number of cells whose antioxidant defence systems

remain healthy enough to cope with growth in a fully oxygenated atmosphere, while the increase in counts observed under ROS-neutralised conditions (whether achieved either by a combination of aerobic processing on a pyruvate-supplemented medium with subsequent transfer to an anaerobic jar, or by anaerobic processing and incubation on pre-reduced unsupplemented medium entirely under anaerobic conditions) represents that fraction of cells whose antioxidant defence systems are unable to cope under conventional aerobic conditions. In the absence of any other stressors, the difference between the initial count and the ROS-neutralised count on such a non-selective medium may thus provide a measure of the number of cells whose metabolic processes have been damaged to the point where they are no longer able to replicate, which is generally regarded as a measure of their loss of viability (Barer *et al.* 1993; Barer and Harwood, 1999), though there are others who believe that such cells may enter the viable but non-culturable (VBNC) state (e.g. Alexander *et al.*, 1999; Grey and Steck, 2001) as discussed in Chapter 1. Applying these principles to the data in Figs. 3.1 and 3.2, the similar counts obtained under aerobic and ROS-neutralised conditions at the outset of the experiment shows that the cells were initially healthy (Fig. 3.1a), and mostly remained so on storage for 6 h–24 h in the earthern vessel (Fig. 3.2a-b). In contrast, the number of healthy cells of *E. coli* NCTC8912 remaining after storage for 6 h in water kept in a brass mutka (Fig. 3.1b) dropped to around 0.01% of the initial value, while approximately 10% of the remaining fraction were sub-lethally injured, with around 90% of the cells being irreversibly inactivated (Fig. 3.16). Since similar results were obtained with copper and brass, but not with earthern, stainless steel, plastic, or glass vessels (Table 3.1), it would seem appropriate to suggest that these effects were due primarily to the dissolution of heavy metals into the water during storage in copper and brass mutkas.

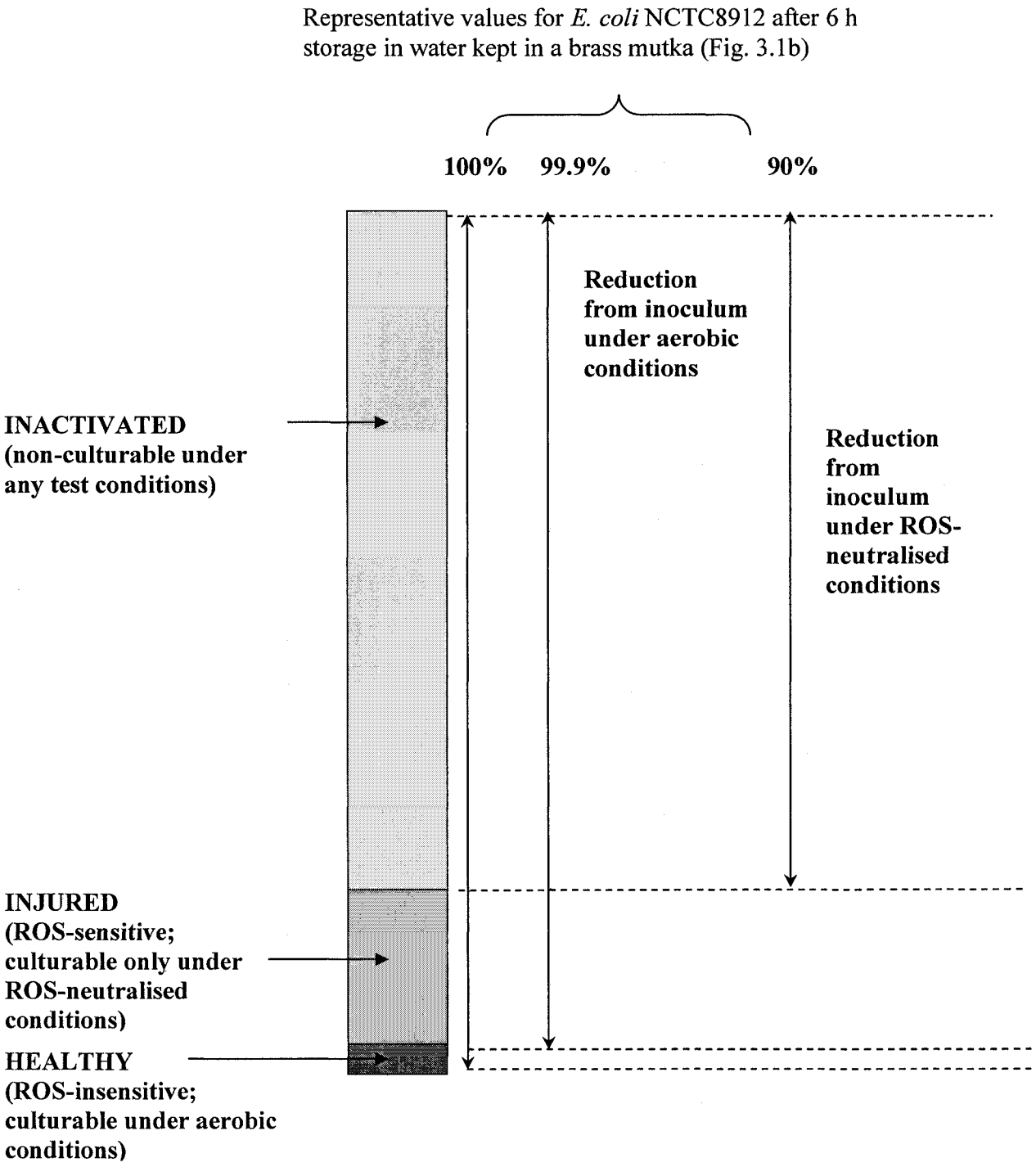


Fig. 3.16 Diagrammatic representation of the relative proportion of inactivated, injured and healthy bacterial cells following exposure to environmental stress (e.g. in water stored in brass mutka).

Heat-treated cells of *E. coli* have been shown to give reduced counts when grown aerobically, in contrast to the increased counts obtained on culture of such cells entirely under anaerobic conditions (e.g. Bromberg *et al.,* 1998). The studies of George and Peck (1998) and George *et al.* (1998) on the effect of oxygen concentration and redox potential on the recovery of sub-lethally heat-damaged cells of *E. coli* O157, *Salmonella enteritidis* and *Listeria monocytogenes* support the concept that growth of injured bacteria under anaerobic conditions can give higher counts than under aerobic conditions. These researchers observed that bacterial heat resistance was greater when enumerated under fully anaerobic conditions, whereas conventional aerobic enumeration suggested an apparent heat resistance that was far lower, with obvious implications for the thermal processing of foodstuffs (Peck and George, 1999).

The growth of *E. coli* inhibited by oxidants has been reported to increase under aerobic conditions when the redox potential of the medium is decreased by adding dithiothreitol, DTT (Bagramyan *et al.,* 2000; Kirakasyan *et al.,* 2004). George and Peck (1998) also noted an increase in count of heat-injured bacteria under aerobic conditions when the redox potential of the medium was lowered by this reducing agent. Such findings are in agreement with the results obtained in Fig. 3.3 where addition of DTT to growth medium without pyruvate increased counts of bacteria kept in brass vessels compared to their conventional aerobic counterparts. However, under anaerobic conditions in pyruvate-supplemented medium DTT gave no further enhancement to the counts and this compound was therefore not used in subsequent experiments.

In the present study an increase in aerobic count was also noticed for *E. faecalis* NCTC775 when the peroxide quencher sodium pyruvate was added (Fig. 3.12) which

was further enhanced under fully ROS-neutralised conditions, although to lesser extent obtained for *E. coli* NCTC8912 (c.f. Fig. 3.1b). In contrast to the present findings, recent research on the enumeration of so-called VBNC cells of *E. faecalis* reported no increase in count when pyruvate or catalase were incorporated into the growth medium (Lleo *et al.,* 2001, 2005), which may be due to the different strains and/or media used or, possibly, because the incorporation of pyruvate (at 0.2%; Lleo *et al.,* 2001) alone is insufficient to fully enumerate injured cells which may grow only when all sources of ROS are neutralised (Fig. 3.12).

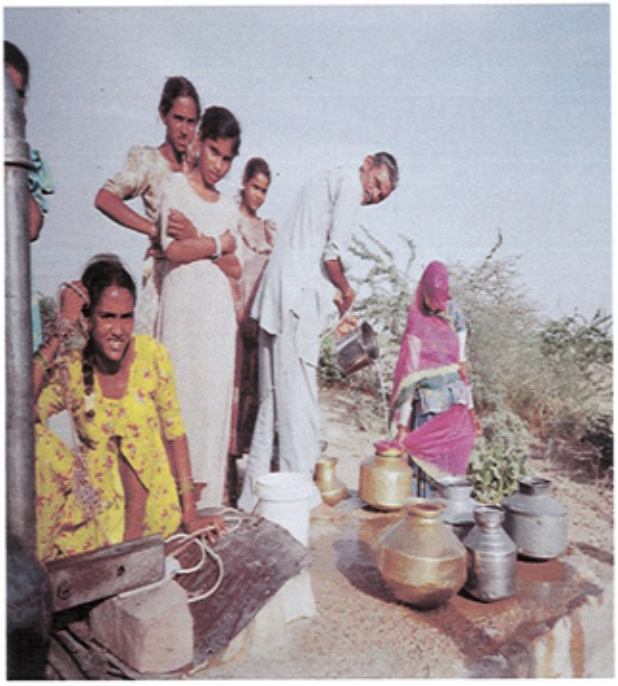
The positive effects of added pyruvate on the counts of four different strains of *E. coli* exposed to brass observed in the present study contrast with the recent observations of Grey and Steck (2001), who found no increase in the plate counts of *E. coli* ED8739 following exposure to 0.5 mmol L^{-1} copper sulphate when the growth medium was supplemented with 0.32 % w/v sodium pyruvate. While such differences may be explained in part by the use of different strains and experimental conditions, their evidence that such cells can be resuscitated simply by rinsing and suspension for several days in a copper-free 0.9% w/v NaCl solution is also consistent with the idea that metal-treated cells are not inactivated but are sub-lethally damaged to the point where they are unable to grow on agar-based media under conventional aerobic conditions. Our results are also in agreement with their observation that "current growth-based microbiological methods for assaying toxicity result in an undercount of the number of viable cells" (Grey and Steck, 2001), though our interpretation favours the respiratory self-destruction hypothesis (Bloomfield *et al.*, 1998), based predominantly on peroxide sensitivity (Bogosian *et al.*, 2000), rather than the notion of a distinct and separate VBNC state.

Domek *et al.* (1984) performed experiments in which coliforms were exposed to dissolved copper ions and they reported that up to 90% of these bacteria showed sub-lethal damage, as demonstrated by their lack of growth on a selective medium containing 0.1% w/v sodium deoxycholate, in contrast to the equivalent non-selective medium. Similar findings have also been obtained by other researchers, showing that coliform bacteria, including *E. coli*, typically fail to grow on selective media following sub-lethal injury or environmental stress (e.g. Bissonnette *et al.,* 1974, Campher *et al.* 1979; Singh and McFeters, 1986; Singh *et al.,* 1986; Kang and Siragusa, 1999). The results of the present study can be interpreted in a similar manner, with *E. coli*, *E. faecalis* and coliforms exposed to copper and zinc as a result of storage in water kept in a brass vessel for 6 h -24 h showing decreased counts on certain selective media, especially in the presence of sodium lauryl sulphate for *E. coli* in mLSA medium (Figs. 3.7b, 3.8a, b and c; Table 3.2) and bile salts in the case of *E. faecalis* in MacConkey number 2 agar (Figure 3.14).

It is known that most selective agents for coliforms contain surfactants (Sartory, 1995), and toxicity in such media may be mediated through membrane damage, as evidenced by disruption to membrane-associated metabolic functions, especially respiration (Domek *et al.,* 1987) and solute transport (Sunda and Huntsman, 1998), providing a plausible explanation for the reductions in counts on selective media observed for different strains of *E. coli* in Figs. 3.6b, 3.7 a, b and c and Table 3.2. A major mechanism of inactivation of bacteria by copper is thought to be by binding to essential biomolecules (Thurman and Gerba, 1989; Straub *et al.,* 1995) and inhibiting general enzyme activity (Yahya *et al.,* 1990), which is also consistent with the present findings that short-term exposure causes sub-lethal injury but that longer-term exposure results

in the inactivation of bacteria. Thus incubation of *E. coli* and *E. faecalis* in water kept in a brass mutka led to cells being undetectable after 48 h, even under ROS-neutralised conditions, which indicates that prolonged storage in a brass vessel appeared to completely inactivate the bacteria, in agreement with earlier reports based on standard aerobic enumeration (e.g. Patwardhan, 1990). Experiments carried out by storing water for 48 h in the brass mutka, transferring the water to a glass vessel and then seeding it with *E. coli* also showed inactivation, with sub-lethal injury evident at 6 h (Plate 3.2), giving undetectable counts within 24 h and thereby demonstrating that the effect is due to the solubilisation of copper and/or zinc into the water, albeit at very low levels.

A recent study on faecal contamination of municipal water supplies at collection points and during storage in urban areas of a South Indian town concluded that water stored in brass vessels was less contaminated with faecal indicator bacteria when compared with water from other storage vessels such as plastic, aluminium, stainless steel and earthern mutkas (Brick *et al.,* 2004). It is also notable that villagers increasingly favour plastic or stainless steel mutkas over brass or copper because they are cheaper to purchase and are more durable than the more traditional materials (Brick *et al.*, 2004), though brass mutkas are still used in some villages in India (Plate 3.3). However, neither plastic nor stainless steel vessels showed a significant reduction in the count of *E. coli* over a 24 h period (Table 3.1) in the present study. Laboratory experiments carried out with water seeded with *E. coli* also supported the results obtained in the field study, with inactivation over a 48 h period under laboratory conditions. However, in practice, the villagers whose brass mutkas were sampled in the present study do not routinely store their water for this period of time, but use the collected water within 24 h.



(a)

(b)

Plate 3.3 Water collection and storage vessels in use in India with photographs taken in Rajasthan (a) shows brass, cast iron and stainless steel vessels, (b) shows a women pouring water from a brass mutka to an earthern vessel, through a cloth filter, to remove the zooplankton that carry Guinea worm.

While Table 3.2 shows that overnight storage (12-15 h) led to a reduction in coliform count, it was not sufficient to completely inactivate these bacteria. Assuming that such results are also applicable to pathogenic bacteria (Clesceri *et al.*, 1998), the implications are that overnight storage in a brass vessel does not necessarily render the water safe to drink, though it may improve its microbiological quality. Plate 3.3 shows some of the different types of storage vessels used in India.

Faecal bacteria become stressed or injured in waters and wastewaters and may then be incapable of growth and colony formation under standard aerobic conditions because of the selective agents used in media for their enumeration (McFeters *et al.*, 1982; Rompre et al., 2002). As a result, many of these damaged bacteria may not be detected and this may produce false-negative results for the bacteriological quality of water (e.g. McFeters *et al.*, 1986). Culturability and virulence in *Salmonella typhimurium* is changed by long-term starvation leading to a decrease in counts on selective MSRV agar as compared to non-selective R2A medium (Reasoner and Geldreich, 1985; Mossel and Struijk, 2004) and nutrient agar (Lesne *et al.,* 2000). Non-selective and low-nutrient R2A minimal medium minimizes the "substrate shock" for nutrient-stressed organisms as compared to standard plate count agar or selective agars for environmental samples (Robinson *et al.,* 1995; Leclerc and Moreau, 2002). The variation in count of brass-stored *E. coli* NCTC8912 on media with different nutrient status under aerobic conditions is likely to be the result of oxidative stress resulting from the respiratory metabolism of sugars and other readily-utilized organic compounds which in an enriched medium is likely to quickly generate a large amount of ROS that is then lethal to the cells (Aldsworth *et al.*, 1999), whereas a low-nutrient medium would be expected to result in a lower rate of respiratory activity and,

consequently, to reduced self-destruction under aerobic conditions (Mackey and Derrick, 1986; Stephens *et al.,* 2000). Thus under ROS-neutralised conditions (e.g. incubation of pyruvate-supplemented growth media in an anaerobic jar or using pre-reduced media with or without pyruvate supplementation in an anaerobic cabinet) similar counts were obtained for *E. coli* on nutrient-deficient and nutrient-rich media (Fig. 3.5), supporting this proposal. In contrast, *E. faecalis* NCTC775 (Figure 3.12) gave a different response to the nutrient status of the enumeration media, with nutrient-rich brain-heart agar and standard nutrient agar giving a higher count than nutrient-deficient R2A medium. *E. faecalis* is a fastidious micro-organism and it is an aerotolerant anaerobe (Flauhat *et al.,* 1998; Rince *et al.,* 2000). It is unable to grow well on nutrient-deficient media because of the nutrient-limiting status of the enumeration media. Also, it does not possess respiratory metabolism and therefore is not susceptible to respiratory self-destruction on rich media, such as brain-heart agar, which may account for the different results, compared with *E. coli.*

The results of the present study clearly demonstrate that the majority of the cells of a laboratory culture of *E. coli* and *E. faecalis* may become sub-lethally injured, rather than killed, by short-term incubation in water kept in a brass vessel (Figs 3.1-3.11; 3.12-3.15). The data for household mutkas shown in Table 3.2 indicate how misleading 'false-negative' coliform counts might be obtained for particular combinations of selective media and growth conditions; thus mLSA medium incubated aerobically gave no detectable coliforms in water kept overnight in a brass mutkas on seven occasions when these bacteria were shown to be present in numbers of up to a thousand or more per 100 mL when enumerated using MacConkey medium under ROS-neutralised conditions. Such findings have obvious implications for the detection and enumeration

of metal-stressed coliforms and *E. coli* in environmental water samples and this theme is developed further in subsequent chapters (e.g. Chapters 6 and 7).

The present study indicates that for maximum enumeration of metal-stressed coliforms, *E. coli* or *E. faecalis*, counts should be performed under ROS-neutralised conditions, either using fully anaerobic conditions with pre-reduced growth medium, or using an anaerobic jar along with pyruvate-supplemented medium. However, even under such conditions, the inhibitory effects of some selective media can still lead to a reduced count (e.g. Figs. 3.7 and 3.14) and careful selection of an appropriate enumeration medium is required, to minimize such inhibition. Such anaerobic effects during enumeration should not be confused with the known enhancement of metal ion toxicity during exposure under anaerobic conditions, e.g. where cupric ions (Cu^{++}) are converted to the more toxic cuprous (Cu^{+}) form (Beswick *et al.*, 1976; McBrien, 1980).

Chapter 4

Comparative inactivation of faecal indicator bacteria in response to environmental stresses

4.1 Introduction

As discussed in Chapters 1 and 3, elevated levels of faecal indicator bacteria in drinking water indicate either, (i) a contaminated source, (ii) inadequate treatment or (iii) post-treatment deficiencies (Domek *et al.*, 1984). Regular monitoring of drinking water sources for faecal indicator bacteria is therefore an essential part of the procedures involved in monitoring the quality of water supplies (Mossel and Struijk, 2004). However, these bacteria are commonly found in samples of contaminated water where they undergo a variable amount of growth, injury or inhibition, due to their exposure to different biological and physico-chemical factors (Chapter 1, section 1.9; Clesceri *et al.*, 1998). These injured bacteria may lose the ability to grow on growth media in routine use, for example selective media, which are otherwise satisfactory for cultivation of healthy cells (Rizzo *et al.,* 2004; Spiegeleer *et al.,* 2004). As an example, heat-injured *E. coli* O157 cannot grow on selective MacConkey sorbitol agar (Kang, 2002). The difference obtained in bacterial counts between non-selective and selective media has been used to describe the extent to which such bacteria have been subjected to stress (e.g. Andrews and Ray, 1989; McCleer and Rowe, 1995). Stress is a physiological response produced in a bacterial cell as a result of a stimulus or because of a stressor. This stimulus or stressor, for example irradiation by short wavelength UV-A rays of sunlight (Fujiioka *et al.*, 1981; Reed *et al.*, 2000; Reed, 2004; Khaengraeng and Reed, 2005), antimicrobial chemicals such as chlorine, chlorine dioxide (Davies and Mazumder; 2003; Rizzo *et al.*, 2004), heat (Hurst *et al.*, 1976; Czechowicz *et al.,* 1996; Mizunoe *et al.,* 1999), or cold (Bogosian *et al.,* 2000), heavy metals (Calabrese and Bissonnette, 1990b; Grey and Steck, 2001) and interactions with other organisms

(Christoffersen *et al.*, 1995) are predominantly present in natural habitats such as the environmental waters into which faecal indicator bacteria are discharged in sewage. The detection of these stressed bacteria using conventional (selective) culture procedures might lead to underestimation of their actual levels in environmental samples such as drinking water, resulting in a significant problem in accurately estimating their numbers (Kang and Siragusa, 1999; Rompre *et al.*, 2002).

Various researchers have attempted to explain the consequences of stress induced in bacterial cells through different hypotheses e.g. sub-lethal doses of stress may cause bacteria to undergo respiration-induced death (self-destruction) when cultured under aerobic conditions, due to the production of reactive oxygen species (ROS) which they are unable to detoxify (Dodd *et al.*, 1997; Aldsworth *et al.*, 1999). An inhibition of growth induced by stress in a bacterial cell can create an imbalance between catabolic and anabolic processes. One explanation for the enhanced resistance acquired by stationary phase bacterial cells going through these detrimental conditions is that these cells possess a less active catabolic metabolism than exponentionally growing cells (Aertsen and Michielis, 2004). Mild doses of stressful environmental conditions subjected to rapidly growing and respiring bacterial cells may lead to an arrest of cell growth, but their respiratory metabolism may continue, producing a burst of free radicals which may be more harmful for bacterial the cell than the original stress itself (Dodd *et al.*, 1997; Reed, 2004).

In contrast, under other circumstances bacterial cells exposed to stressful conditions may induce a protective stress response triggered by extracellular signaling factors, which may enable unstressed cells to survive or become resistant: for example, exposure of bacteria such as *Streptococcus mutans* to low pH produces extracellular induction components which maybe transmitted to other unstressed cells through a cell-cell signaling (Li *et al.*, 2002). Aerobically growing bacteria such as *Escherichia coli* and *Salmonella typhimurium* maintain a defence system for each stress response, for example in response to high levels of H_2O_2 that may lead to the oxidative stress, the peroxide stimulon regulated by the regulatory protein OxyR (gene locus *oxyR*) is induced (Farr and Kogama, 1991; Droge, 2002). In *Escherichia coli* four stress-mediated systems are induced in response to different conditions such as environmental stress, nutrient-limiting conditions, and toxic chemical or physical agents. These are the heat shock (*htpR*- controlled) regulon, the oxidative stress (o*xyR*-controlled) regulon, the SOS (*lexA*-controlled) regulon, and the stringent response regulon. As an example, UV-irradiation causing DNA damage to bacterial cells induces the production of both the heat shock response and the SOS response (van Bogelen *et al.*, 1987; Chang *et al.*, 2002).

As previously stated, conventional growth media may be effective in culturing and enumerating healthy cells but physiologically stressed cells may not be able to grow on these media. This is supported by the fact that different researchers have incorporated peroxide-degrading compounds such as sodium pyruvate (Czechowicz *et al.*, 1996) or enzymes such as catalase (Mackey and Seymour, 1987) in agar-based growth media,

resulting in higher counts when compared with medium without such additives. Other workers have achieved rehabilitation by growing stressed bacteria under anaerobic conditions, for example, *E. coli* O157 cells subjected to heat gave a higher count in growth media incubated under anaerobic conditions as compared to aerobic incubation (Murano and Pierson, 1993; Bromberg *et al.*, 1998). Such observations support the proposal that ROS produced in stressed cells (Aldsworth *et al.*, 1999) or due to oxidation of medium components (Stephens *et al.*, 2000) may inhibit the growth of sub-lethally injured cells of bacteria under aerobic conditions, rather than being caused directly by the stress itself.

Inactivation and injury of bacteria by various individual factors has been studied for over a century. Several researchers have suggested that sunlight is one of the most important factors responsible for the inactivation of faecal bacteria in fresh water (e.g. Barcina *et al.*, 1990) and in sea water (e.g. Davies and Evison, 1991; Sinton *et al.*, 1999; 2002). Other researchers have shown that the chemical disinfectant chlorine, widely used for the treatment of water, can induce injury by exerting disruptive effects on a variety of sub-cellular components and metabolic processes such as respiration (Lisle *et al.* 1999). Temperature and pH are two other environmental factors affecting growth, and have been studied mostly because of their importance in fundamental research and their practical significance in natural environments (Donald and Kotrola, 1995; Rosso *et al.*, 1995; Diez-Gonzalez and Russell, 1999; Gnanou-Besse, 2002; Ross *et al.*, 2003). Starvation of bacterial cells has been studied with respect to survival and long-term preservation in water (Liao and Shollenberger, 2003; Gerbeth *et al.*, 2004). Recent

research has also highlighted the fact that inactivation of enteric micro-organisms such as *E. coli* and *Enterococcus faecalis* may be enhanced by using a combination of chlorine disinfection with UV treatment for the disinfection of water, rather than using a single treatment method (Koivunen and Heinonen-Tanski, 2005). This Chapter describes experiments carried out to investigate the effects of a number of environmental factors on the enumeration of *E. coli* and *Enterococcus faecalis*, using ROS-neutralised conditions and various selective and non-selective media to provide information on the extent of injury and/or inactivation for each factor.

The specific objectives of the studies described in the Chapter were:

1. To investigate the effects of various environmental factors, i.e. sunlight, temperature, hypochlorite disinfectant, low pH and starvation on the counts of laboratory strains and environmental isolates of the faecal indicator bacteria such as *Escherichia coli* and *Enterococcus faecalis*, using non-selective and selective media to find out which types of media and growth conditions give the highest and lowest counts for stressed micro-organisms.

2. To investigate the effects of treatments that neutralize reactive oxygen species (e.g. using sodium pyruvate to neutralize hydrogen peroxide, or using anaerobic growth conditions to eliminate respiration-based by-products of oxygen), by comparing counts of laboratory strains and environmental isolates of *Escherichia coli* and *Enterococcus faecalis* damaged by different stresses and enumerated on growth media with and without such neutralizing reagents and grown under aerobic and anaerobic conditions.

Essentially, this Chapter extends the work of the previous Chapter (on stress resulting from storage in brass/copper vessels) to other environmental stresses.

4.2 Materials and Methods

Cell suspensions of bacterial cultures of *Escherichia coli* and *Enterococcus faecalis* were prepared as described in Chapter 2 and subjected to individual factors in the laboratory, as described below.

4.2.1 Effects of UV-A radiation and natural sunlight on the enumeration of faecal indicator bacteria

In this study two light sources were used to induce stress in bacterial cells, namely (i) UV-A radiation and (ii) natural sunlight. The UV-A source, used in the UK at Northumbria University, consisted of a cabinet containing three Phillips 40W-R UV-A sunlamp tubes (Philips Lighting, Croydon, UK), two small ventilation fans attached at both ends of the cabinet (to control temperature) and aluminium foil lined walls (to reflect light within the cabinet). Bacterial suspensions in tubes or transparent bottles were placed in the middle of the cabinet and exposed to a UV-A intensity of approximately 20W m^{-2}. In India, bacterial cell suspensions were exposed to full strength natural sunlight in the laboratory studies at Panjab University, Chandigarh. The cell suspensions were poured into 1 L transparent universal bottles, which were placed on the roof of Panjab University, Microbiology Department during the peak period for full-strength sunlight (10 a.m. - 4 p.m). For both light sources, timed samples, i.e. at 0, 2, 4 and 6 hour were taken from the illuminated bottles and a control that was kept in the dark. Samples were processed in dim light by ten-fold serial dilution and then spread plated using the Miles and Misra droplet method as described in Chapter 2 on various selective and non-selective media supplemented with and without 0.05% w/v sodium

pyruvate (0.5 g L^{-1}). The plates were incubated at 37°C either for 24 h under standard aerobic conditions or for 48 h under ROS-neutralised conditions as detailed in Chapter 3.

4.2.2 Effects of high temperature exposure on the enumeration of faecal indicator bacteria

A volume of 20 mL of cell suspensions was poured into a sterile universal bottle and either heat-treated by exposure to a temperature of 52°C to 54°C in a water-bath, or kept at room temperature, 25°C, as a control. Timed samples (0, 15, 30, 45, 60 and 90 minutes) from both bottles were enumerated on different growth media, i.e. various selective and non-selective media supplemented with and without 0.05% w/v sodium pyruvate (0.5 g L^{-1}) by ten-fold serial dilutions using the Miles and Misra droplet method. Plates were incubated at 37°C under aerobic and ROS-neutralised conditions, as described in Chapter 2 and 3.

4.2.3 Effects of sodium hypochlorite on the enumeration of faecal indicator bacteria

A volume of 10 mL of 12% w/v of sodium hypochlorite stock solution was prepared freshly each time it was required, and diluted to give working solutions with free chlorine levels between 1.0 mg L^{-1} and 0.1 mg L^{-1}: the free chlorine content was established by titration using the N, N-diethyl-p-phenylendiamine (DPD) ferrous titrimetric method (Clesceri *et al.* 1998). Measured free chlorine levels of the working solutions were found to be 0.9 μg mL^{-1}, 0.6 μg mL^{-1} and 0.3 μg mL^{-1} in the experimental solutions. Then a volume of 100 μL of bacterial cell suspension was added into two

universal bottles with the first bottle containing 10 mL of quarter-strength Ringer's solution (control) and the second bottle containing 10 mL of the working solution of sodium hypochlorite. Timed samples (0, 15, 30, 45, 60 and 120 minutes) were taken after exposure to hypochlorite and processed by ten-fold serial dilutions in the Ringer's solution containing 2.5% (w/v) sodium thiosulphate solution, to neutralize any free chlorine left after contact with the bacterial suspension. Surface spread plating was performed using the Miles and Misra droplet method on to non-selective and various selective media. Plates were incubated at 37 °C under different sets of growth conditions (aerobic and ROS-neutralised), as described in Chapters 2 and 3.

4.2.4 Effects of low pH on the enumeration of faecal indicator bacteria

A volume of 1 mL of each bacterial cell suspension was suspended into two bottles, with the first bottle containing 9 mL of quarter-strength Ringer's solution (control; pH $\approx$ 7) and the second bottle containing distilled water adjusted to pH = 2 with dilute hydrochloric acid. The pH value was confirmed using a pH meter (Hanna Instruments Limited, Leighton Buzzard, UK). Samples were taken after regular intervals of time, i.e. at 0, 3, 6 and 24 h. They were processed by serial decimal dilution and enumerated on different growth media and incubation conditions, as described in Chapters 2 and 3.

4.2.5 Effects of long-term incubation in sterile distilled water on the enumeration of faecal indicator bacteria

A volume of 1 mL of the bacterial cell suspension was inoculated into foil-covered glass flask containing 100 mL of sterile distilled water. Initial counts of bacteria were determined at 0 h using ten-fold serial dilutions with spread plating on nutrient agar supplemented with and without 0.05% w/v of sodium pyruvate under aerobic and ROS-neutralised conditions described in Chapters 2 and 3. The sample was then stored at 25°C and 37°C. Samples were taken regularly every fifteen days from both treatments and enumerated on non-selective and various selective media incubated under different sets of growth conditions, described in Chapters 2 and 3.

4.3 Results

4.3.1 Effects of UV-A radiation and natural sunlight on the enumeration of faecal indicator bacteria

Escherichia coli: **enumeration of different strains following exposure to UV-A and sunlight** Table 4.1 shows the mean plate counts for preliminary experiments conducted using *E. coli* NCTC8912 kept in dark as the control and after exposure to UV-A radiation for up to 4 hours. The bacterial cell count in CFU mL^{-1} displays the mean value obtained with the upper and lower 95% confidence limits shown in brackets, below each mean value. Enumeration was carried out using nutrient agar in four sets of growth conditions, i.e. standard aerobic conditions (unsupplemented medium), peroxide-neutralised conditions (0.05% sodium pyruvate-supplemented medium), anaerobic condition (in an anaerobic jar) and ROS-neutralised conditions (incubation in an anaerobic jar of the medium supplemented by 0.05% sodium pyruvate) with incubation at 37°C. The results indicate that exposure to UV-A caused a rapid decrease in counts reaching undetectable levels (detection limit 50 mL^{-1}) within 4 h for all four growth conditions. Exposure to UV-A for 3 h showed that the unsupplemented nutrient agar under aerobic conditions gave the lowest value followed by the same medium incubated under anaerobic conditions. The peroxide-neutralised conditions created by inclusion of sodium pyruvate in the growth medium showed increases in count compared with the corresponding count obtained for aerobic and anaerobic unsupplemented media, while the ROS-neutralised conditions created by inclusion of sodium pyruvate in the growth medium with anaerobic incubation gave the highest count. In contrast, the control even

after 4 h showed no substantial change in overall count under all four sets of growth conditions, exhibiting no indication of any sub-lethal injury (Table 4.1).

Table 4.1 Effect of UV-A radiation on the enumeration of *E. coli* NCTC8912 using nutrient agar Mean counts are shown (upper and lower 95% confidence limits in brackets below each mean value) for *E. coli* exposed to UV-A for up to 4 h, incubated either aerobically (unsupplemented medium), or aerobically with supplementation of the medium with 0.05% w/v sodium pyruvate (peroxide-neutralised conditions), anaerobically (anaerobic jar), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised conditions).

		Mean count (CFU mL^{-1})			
UV-A	**Time (h)**	**Aerobic**	**Peroxide-neutralised**	**Anaerobic**	**ROS-neutralised**
0 (control in dark at 25°C)	0	6.0×10^7 (5.4×10^7-6.6×10^7)	5.9×10^7 (5.0×10^7-7.0×10^7)	5.5×10^7 (4.6×10^7-6.7×10^7)	6.2×10^7 (5.9×10^7-6.5×10^7)
	4	4.7×10^7 (4.1×10^7-5.3×10^7)	4.7×10^7 (3.9×10^7-5.8×10^7)	4.4×10^7 (3.9×10^7-5.1×10^7)	4.5×10^7 (3.7×10^7-5.6×10^7)
UV-A					
	1	1.5×10^4 (9.8×10^4-2.3×10^3)	3.4×10^5 (2.6×10^5-4.5×10^5)	2.0×10^4 (1.4×10^4-2.7×10^4)	6.0×10^5 (5.0×10^5-6.9×10^5)
	2	1.6×10^3 (8.6×10^3-3.0×10^2)	5.7×10^3 (3.3×10^3-9.9×10^3)	3.7×10^3 (2.6×10^3-5.2×10^3)	2.5×10^4 (1.7×10^4-3.7×10^4)
	3	2.5×10^2 (2.5×10^2)	7.2×10^2 (1.9×10^3-2.7×10^2)	5.0×10^2 (1.4×10^3-1.8×10^2)	5.0×10^3 (4.0×10^3-6.5×10^3)
	4	<50	<50	<50	<50

Since selective media would be used in subsequent experiments and these might be expected to give lower counts than a non-selective medium, an exposure time of 2 h was selected for UV-A exposure in order to achieve a suitable level of sub-lethal injury. Fig. 4.1 shows plate counts in the form of a bar graph for UV-A-irradiated *E. coli* NCTC8912 obtained for three sets of enumeration conditions, i.e. (i) standard aerobic conditions (pyruvate-unsupplemented), (ii) peroxide-neutralised conditions (pyruvate-supplemented) and (iii) ROS-neutralised conditions (pyruvate-supplemented medium with anaerobic incubation) using non-selective nutrient agar and various selective media, i.e. MacConkey agar, m-FC-R agar, m-Endo agar and mLSA. Overall the counts following UV-A exposure decreased by 50-fold to 900000-fold compared to the initial inoculum, depending upon the type of growth medium and enumeration conditions. Examining the aerobic plate counts, all selective media gave a lower count than nutrient agar with MacConkey agar showing the lowest decrease followed by m-Endo agar, then m-FC-R agar and with the greatest decrease seen in the case of mLSA. Higher counts were seen in all growth media when incubated under peroxide-neutralised conditions with further enhancements seen under ROS-neutralised conditions when compared with the equivalent aerobic conditions. In the case of non-selective nutrient agar there was an approximately 10-fold increase in aerobic count when enumerated under ROS-neutralised conditions. The various selective media gave more variable increases under ROS-neutralised conditions, with m-Endo agar showing the lowest increase and mLSA the greatest increase (compared to the equivalent aerobic count) when incubated under ROS-neutralised conditions.

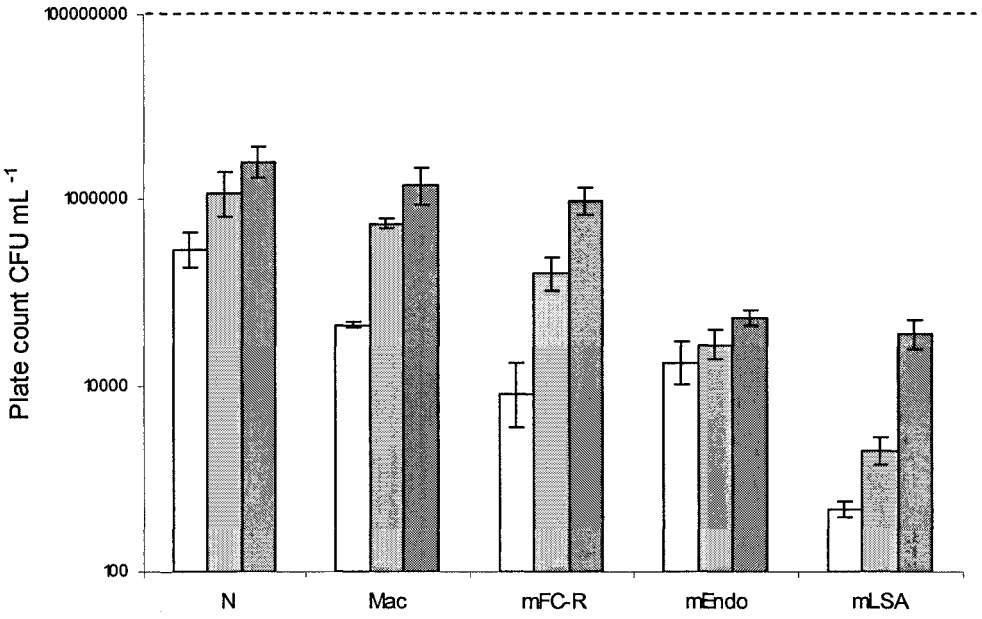


Figure 4.1 Effect of enumeration conditions on *E. coli* NCTC8912 after 2 h UV-A exposure enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, and m-FC agar without rosolic acid (mFC-R), incubated either aerobically in unsupplemented medium (unshaded bars), or peroxide-neutralised with aerobic incubation in medium supplemented with 0.05% w/v sodium pyruvate (light-shaded), or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 4.2 a, b, and c illustrates results obtained for 3 other strains of *E. coli* using the same range of growth media but enumerating under only two sets of conditions, i.e. standard aerobic conditions (pyruvate-unsupplemented medium) and ROS-neutralised conditions (pyruvate-supplemented medium with anaerobic incubation), using natural sunlight as a stressor instead of UV-A. Fig. 4.2a provides data for *E. coli* TN675 using an exposure time of 2 h in natural sunlight, while Fig. 4.2b and Fig. 4.2c shows corresponding results for the two Indian isolates, *E. coli* PUCC061 and PUCC113 using a sunlight exposure time of 4 h, (since 2 h exposure to full-strength sunlight gave minimal changes in the count of these environmental isolates in preliminary experiments; data not shown). The broad patterns of results in Fig 4.2 a-c are similar to those seen in Fig. 4.1 for *E. coli* NCTC8912. Comparing the increase in count of non-selective nutrient agar under aerobic and ROS-neutralised conditions, a 10-fold increase in ROS-neutralised count was observed for *E. coli* TN675 from its corresponding aerobic count in Fig. 4.2a; while the environmental isolates *E. coli* PUCC061 (Fig. 4.2b) and PUCC113 (Fig. 4.2c) showed a somewhat lower increase (3-4 fold) under ROS-neutralised conditions. In comparing the selective media under aerobic conditions, MacConkey agar in all three cases gave the highest counts followed by m-Endo agar, m-FC-R agar and with m-LSA giving the lowest count in all cases. An increase in count compared to the equivalent aerobic value was noted for all selective media when incubated under ROS-neutralised conditions, though this was generally smallest for m-Endo.

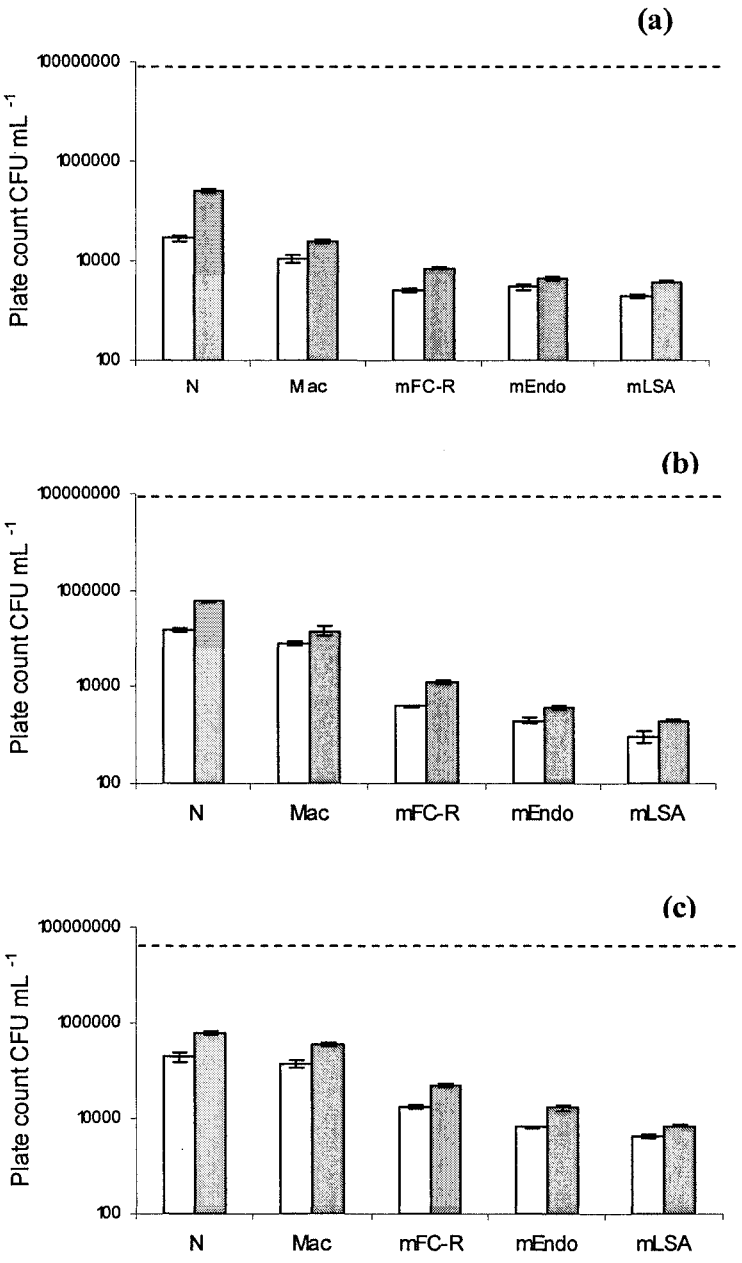


Figure 4.2 Effect of enumeration conditions on three strains of *E. coli* after UV-A exposure (a) *E. coli* TN675 after 2 h exposure to UV-A; (b) *E. coli* PUCC061 after 4 h exposure to UV-A; (c) *E. coli* PUCC113 after 4 h exposure to UV-A, enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, and m-FC agar without rosolic acid (mFC-R), incubated either aerobically in unsupplemented medium (unshaded bars), or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

***Enterococcus faecalis:* enumeration of different strains following exposure to UV-A and sunlight** Fig. 4.3 shows results in the form of bar graph for *E. faecalis* NCTC775 exposed to UV-A irradiation for 4 h and enumerated under two sets of growth conditions, i.e. standard aerobic conditions (pyruvate-unsupplemented medium) and ROS-neutralised conditions (pyruvate-supplemented medium with anaerobic incubation). The media used included non-selective nutrient agar, and various selective media, i.e. bile aesculin agar, KF streptococcus agar and Slanetz & Bartley agar and MacConkey agar number 2. Overall after UV-A exposure 100-fold to 3000-fold decreases in count were noted, compared to the initial inoculum. The decreases in count depended upon the growth media and incubation conditions used for enumeration. Under standard aerobic conditions nutrient agar gave highest count followed by Slanetz & Bartley agar, Bile aesculin agar, KF streptococcus agar and MacConkey agar number 2 which acted to be most inhibitory by giving lowest count. Under ROS-neutralised conditions the results for nutrient agar contrasted with those obtained for the *E. coli* strains (Fig. 4.1 and 4.2) by showing a smaller increase in count. In case of selective media KF streptococcus agar showed the greatest increase of almost 15-fold whereas Bile aesculin agar showed an increase of 2.5-fold and Slanetz & Bartley agar and MacConkey agar number 2, of around 2-fold from the corresponding aerobic values. In general the selective media even under ROS-neutralised conditions did not reach the corresponding value for nutrient agar but proved to be less inhibitory for growth of UV-A irradiated *E. faecalis* NCTC775 cells when compared to standard aerobic conditions using selective media.

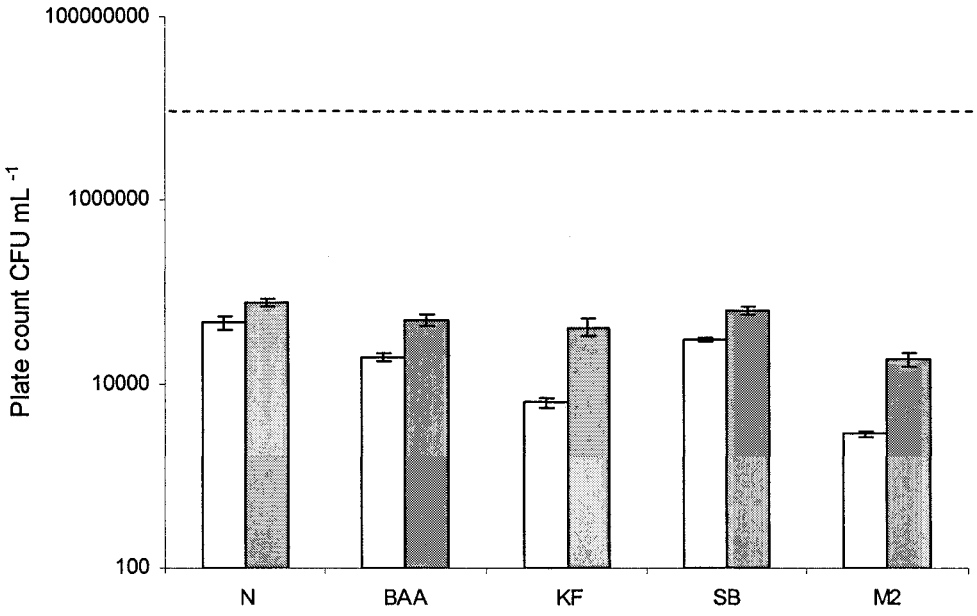


Figure 4.3 Effect of enumeration conditions on *E. faecalis* NCTC775 after 4 h UV-A exposure enumerated on non-selective nutrient agar (N) and various selective media, namely bile aesculin agar (BAA), KF streptococcus agar (KF), Slanetz and Bartley agar (SB), or MacConkey agar number 2 (M2), incubated either aerobically in unsupplemented medium (unshaded bars), or under ROS-neutralised conditions i.e. medium supplemented with 0.05% sodium pyruvate and incubated in an anaerobic jar (dark-shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 4.4a-c represents results for a similar set of experiments performed using three other *E. faecalis* strains, i.e. a laboratory strain ATCC35550 and two Indian environmental isolates, *E. faecalis* PTO1 and PTO2, exposed to natural sunlight for 4 h. Enumeration was carried out using narrower range of growth media but with similar incubation conditions (standard aerobic incubation and ROS-neutralised conditions) as described for *E. faecalis* NCTC775 in Fig. 4.3. Overall the results showed a similar trend as obtained for *E. faecalis* NCTC775 (Fig. 4.3). A somewhat lesser effect of ROS-neutralisation was noted using non-selective nutrient agar compared to its corresponding aerobic value. Comparing the aerobic and ROS-neutralised count on selective media, increases in count were noted for ROS-neutralised conditions with each media giving higher count, these were sometimes also rather small. Describing the results for strain ATCC35550 in Fig 4.4a, plates supplemented with pyruvate and incubated under anaerobic conditions gave increases in count, which was almost 2-3 fold for nutrient agar, Slanetz & Bartley agar and KF streptococcus agar when compared with unsupplemented plates incubated under aerobic conditions. A lesser ROS-neutralised effect was seen for strain ATCC35550 as compared to *E. faecalis* NCTC775 using selective media. The two environmental isolates shown in Fig 4.4b-c also depicted similar sets of results as seen in Fig. 4.4a, where a small increase in counts under ROS-neutralised conditions was obtained on all growth media when compared to the corresponding aerobic value. This variability in results between strain NCTC775 exposed to UV-A and the other three strains exposed to natural sunlight might be due to their different levels of susceptibility and resistance to UV-A radiation, with strain NCTC775 being more sensitive.

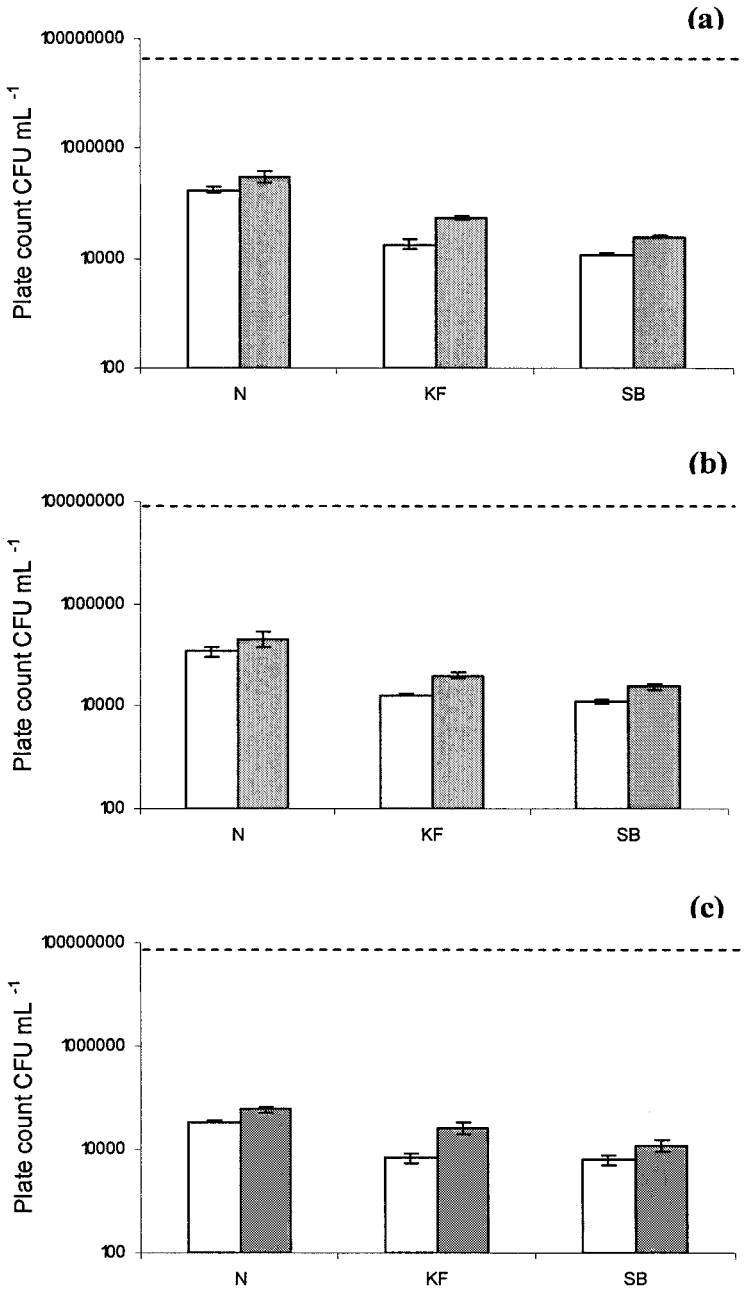


Figure 4.4 Effect of enumeration conditions on three strains of *E. faecalis* after 4 h UV-A exposure (a) *E. faecalis* ATCC35550 (b) *E. faecalis* PTO1, (c) *E. faecalis* PTO2; enumerated on non-selective nutrient agar (N), KF streptococcus agar (KF), or Slanetz and Bartley agar (SB), incubated either aerobically in unsupplemented medium (unshaded bars), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

4.3.2 Effects of high temperatures on the enumeration of faecal indicator bacteria

***Escherichia coli*: effects of elevated temperature** Preliminary experiments were carried out to optimise the exposure time and temperature, which are tabulated for *E. coli* NCTC8912 in Table 4.2, for exposure times up to 60 minutes and temperature in control sample and experimental samples (52°C and 54°C). Four sets of growth conditions were used for enumeration using non-selective nutrient agar, i.e. standard aerobic conditions, peroxide-supplemented conditions, anaerobic condition and ROS-neutralised conditions described in detail in section 4.3.1. The results for all four sets of growth conditions for the control sample showed minimal decreases in cell count, thus indicating no substantial sub-lethal damage. In contrast, a decrease in cell count was observed when compared to the initial inoculum in case of experimental samples exposed to temperatures of 52°C and 54°C. The decreases in cell count when the exposure times of experimental sample were increased, with aerobic conditions enumerating the lowest count. Thus exposure of *E. coli* NCTC8912 to temperature of 52°C for up to 60 minutes gave undetectable counts under aerobic and anaerobic conditions (detection limit 50 mL^{-1}) on unsupplemented nutrient agar plates. However the counts obtained on nutrient agar (pyruvate-supplemented) plates under peroxide-neutralised and ROS-neutralised conditions were higher than corresponding counts on unsupplemented nutrient agar at this time, and also at earlier stages. A broadly similar pattern of results was noted for the experimental temperature of 54°C, with ROS-neutralised counts showing maximum values followed by peroxide-neutralised counts and then aerobic and anaerobic counts using unsupplemented nutrient agar.

Table 4.2 Effects of high temperatures on the enumeration of *E. coli* NCTC8912 using nutrient agar Mean counts are shown (upper and lower 95% confidence limits in brackets below each mean value) for *E. coli* exposed to temperatures up to 54°C, incubated either aerobically (unsupplemented medium), or aerobically with supplementation of the medium with 0.05% w/v sodium pyruvate (peroxide-neutralised conditions), anaerobically (anaerobic jar), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised conditions).

		Mean count (CFU mL^{-1})			
High Tempt	**Time (min)**	**Aerobic**	**Peroxide-neutralised**	**Anaerobic**	**ROS-neutralised**
0 (control in dark at 25°C)	0	4.7×10^7 (4.1×10^7-5.3×10^7)	5.0×10^7 (4.2×10^7-6.1×10^7)	4.9×10^7 (3.8×10^7-6.4×10^7)	5.3×10^7 (4.5×10^7-6.4×10^7)
	60	3.8×10^7 (3.0×10^7-4.9×10^7)	4.0×10^7 (2.9×10^7-5.4×10^7)	3.5×10^7 (2.7×10^7-4.6×10^7)	3.7×10^7 (3.1×10^7-4.4×10^7)
52°C	15	3.0×10^6 (2.0×10^6- 4.0×10^6)	4.6×10^6 (3.7×10^6-5.6×10^6)	3.6×10^6 (2.8×10^6-4.7×10^6)	6.2×10^6 (5.5×10^6-7.0×10^6)
	30	1.2×10^4 (7.0×10^3-2.0×10^4)	4.5×10^5 (3.9×10^5-5.2×10^5)	3.5×10^5 (2.7×10^5-4.6×10^5)	8.0×10^5 (7.3×10^5-8.6×10^5)
	45	1.7×10^3 (1.2×10^3-2.5×10^3)	8.5×10^3 (4.0×10^4-1.8×10^3)	3.0×10^3 (2.4×10^3-3.7×10^3)	3.5×10^4 (2.6×10^4-4.6×10^4)
	60	<50	7.8×10^2 (5.0×10^2-1.2×10^3)	<50	4.0×10^3 (3.0×10^3-5.0×10^3)
54°C	15	1.5×10^4 (8.2×10^3-3.0×10^4)	6.4×10^4 (5.2×10^4-7.8×10^4)	4.2×10^4 (3.6×10^4-4.9×10^4)	1.1×10^5 (4.8×10^4-2.6×10^5)
	30	6.5×10^2 (2.4×10^2-1.7×10^3)	5.5×10^3 (5.0×10^2-6.2×10^3)	1.6×10^3 (6.8×10^2-3.5×10^3)	9.8×10^3 (7.0×10^3-1.4×10^4)
	45	<50	<50	<50	<50
	60	<50	<50	<50	<50

On the basis of results obtained in Table 4.2, an exposure time of 45 minutes at 52°C and 54°C was selected for further experimentation with different strains of *Escherichia coli*. Fig. 4.5 shows plate counts of *E. coli* NCTC8912, enumerated using nutrient agar and various selective media, i.e. MacConkey agar, m-FC-R agar, m-Endo agar and mLSA under three sets of growth conditions, i.e. standard aerobic conditions, peroxide-neutralised conditions and ROS-neutralised conditions. The results clearly indicate that after exposure to a temperature of 52°C for 45 minutes the cell count decreased (300-fold to 86000-fold) compared to initial inoculum. The noted decreases in cell count depended upon growth medium and incubation conditions used for enumeration. Fir non-selective nutrient agar, plates of unsupplemented medium incubated under standard aerobic conditions gave the lowest count. Increases in cell count were noted under peroxide-neutralised conditions for nutrient agar with a further enhancement in count under ROS-neutralised conditions. Comparing the selective media under aerobic conditions, MacConkey gave highest count, while mLSA gave the lowest count, with both mFC-R and m-Endo agar gave intermediate values. Enhancements in count under peroxide-neutralised conditions were noted on each medium except m-Endo, with further increases under ROS-neutralised conditions when compared with aerobic conditions. Overall, non-selective nutrient agar under ROS-neutralised conditions gave approximately 10-fold increase in count when compared to the corresponding count for pyruvate-unsupplemented plates incubated under aerobic conditions. The selective media showed variable increases in plate counts; MacConkey and m-Endo of 3-fold, mLSA of 4-fold and mFC-R of approximately 12-fold increase in count under ROS neutralising conditions when compared with the corresponding aerobic conditions.

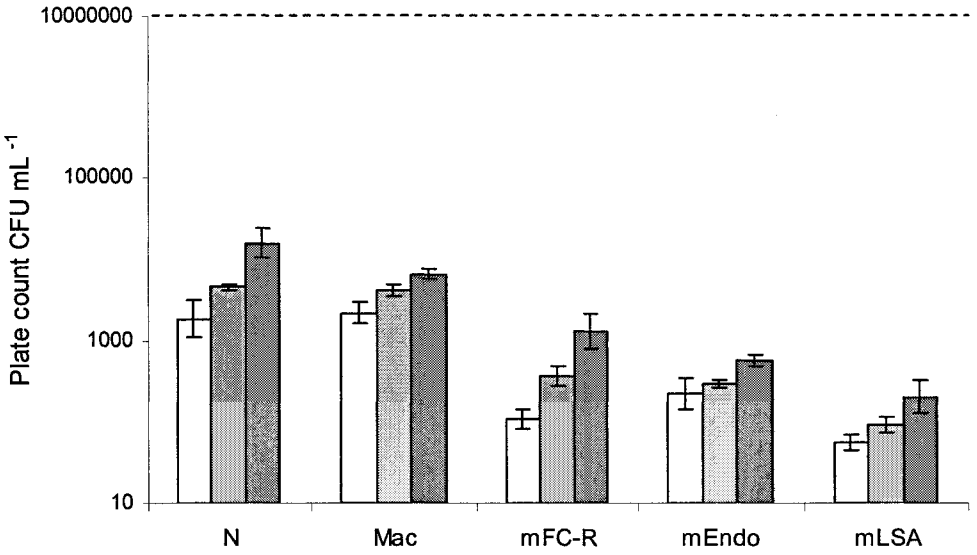


Figure 4.5 Effect of enumeration conditions on *E. coli* NCTC8912 after exposure to high temperature of 52°C for 45 minutes enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, and m-FC agar without rosolic acid (mFC-R), incubated either aerobically in unsupplemented medium (unshaded bars), or peroxide-neutralised with aerobic incubation in medium supplemented with 0.05% w/v sodium pyruvate (light-shaded), or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 4.6a-c describes results for plate counts for a further experiment conducted using a narrower range of two sets of growth conditions, i.e. (i) standard aerobic conditions and (ii) ROS-neutralised conditions using other three strains of *E. coli,* i.e. TN675 (Fig 4.6a) a laboratory strain, and two environmental isolates, i.e. PUCC061 (Fig 4.6b) and PUCC113 (Fig. 4.6c). In the case of the environmental isolates exposure temperature of 54°C for 45 minutes was selected as exposure time of 45 minutes at 52°C resulted in minimal sub-lethal injury (data not shown). These three strains showed broadly similar trends in terms of the changes in cell counts across the media and incubation conditions to those observed for *E. coli* NCTC8912 (Fig. 4.5) but the overall differences were somewhat less than obtained for NCTC8912. The results on non-selective nutrient agar plates incubated under ROS-neutralised conditions gave almost 6-fold higher count in *E. coli* strain TN675 (Fig 4.6a), 4-fold in PUCC061 (Fig 4.6b) and 3-fold PUCC113 (Fig. 4.6c) when compared with standard aerobic plates. In the case of the selective media under aerobic conditions, mLSA proved to be most inhibitory in all three strains (Fig. 4.6) giving the lowest count followed by m-Endo and m-FC-R, whereas MacConkey agar was generally the least inhibitory medium. Under ROS-neutralised conditions the count on all selective media increased but not reaching the level of non selective nutrient agar, with m-FC agar showing the maximum increase in count and m-Endo generally showing the least increase. Overall, MacConkey agar proved to be closest in efficacy to the non-selective nutrient agar medium.

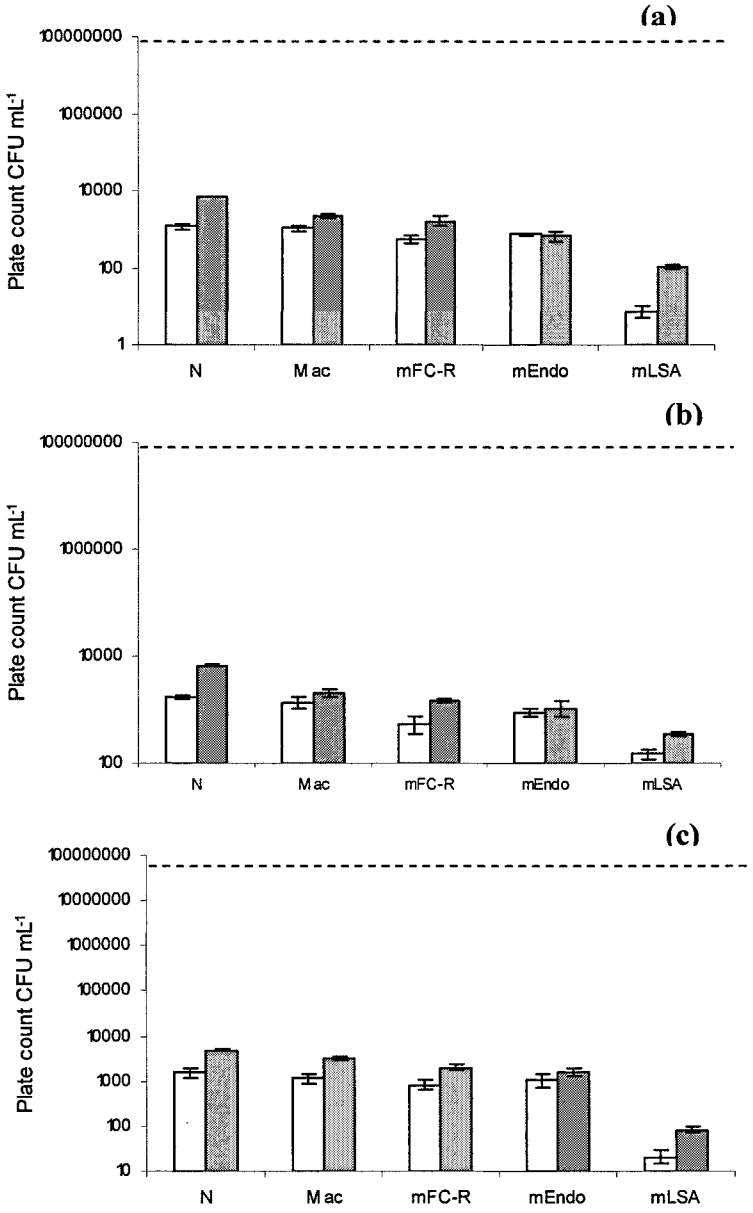


Figure 4.6 Effect of enumeration conditions on three strains of *E. coli* after exposure to high temperature UV-A exposure (a) *E. coli* TN675 after exposure to 52°C for 45 minutes; (b) *E. coli* PUCC061 after exposure to 54°C for 45 minutes; (c) *E. coli* PUCC113 after exposure to 54°C for 45 minutes, enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, and m-FC agar without rosolic acid (mFC-R), incubated either aerobically in unsupplemented medium (unshaded bars) or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

***Enterococcus faecalis:* effects of elevated temperature** Fig. 4.7 provides results for *E. faecalis* NCTC775 exposed to high temperature of 52°C for 90 minutes and enumerated under two sets of growth conditions, i.e. standard aerobic conditions and under ROS-neutralised conditions. The media used for enumeration included non-selective nutrient agar, and various selective media, i.e. bile aesculin agar, KF streptococcus agar, Slanetz & Bartley agar and MacConkey agar number 2, with incubation of agar plates at 37°C for 48 h. After exposure to high temperature there was a substantial decrease in count (200-fold to 100000-fold) when compared with the initial inoculum. The decreases in count were dependent upon growth media and incubation conditions used for enumeration of sub-lethally damaged cells. Describing the results obtained under aerobic conditions, non-selective nutrient agar gave highest count followed by selective Slanetz & Bartley agar and bile aesculin agar giving intermediate values, followed by KF streptococcus agar and lowest count was obtained on MacConkey number 2. Under ROS-neutralised conditions enhancements in count from aerobic conditions were noted. In the case of non-selective nutrient agar a 5-fold increase in count was observed under ROS-neutralised conditions compared to aerobic conditions. Comparing the selective media which showed variable increases, KF streptococcus agar showed the greatest increase of almost 13-fold while bile aesculin agar gave a 6-fold increase, with Slanetz & Bartley agar giving a 4-fold change and MacConkey number 2 giving a 3-fold increase under ROS-neutralised conditions. In general both non-selective and selective media proved to be less inhibitory under ROS-neutralised conditions for *E. faecalis* NCTC775 exposed to high temperature.

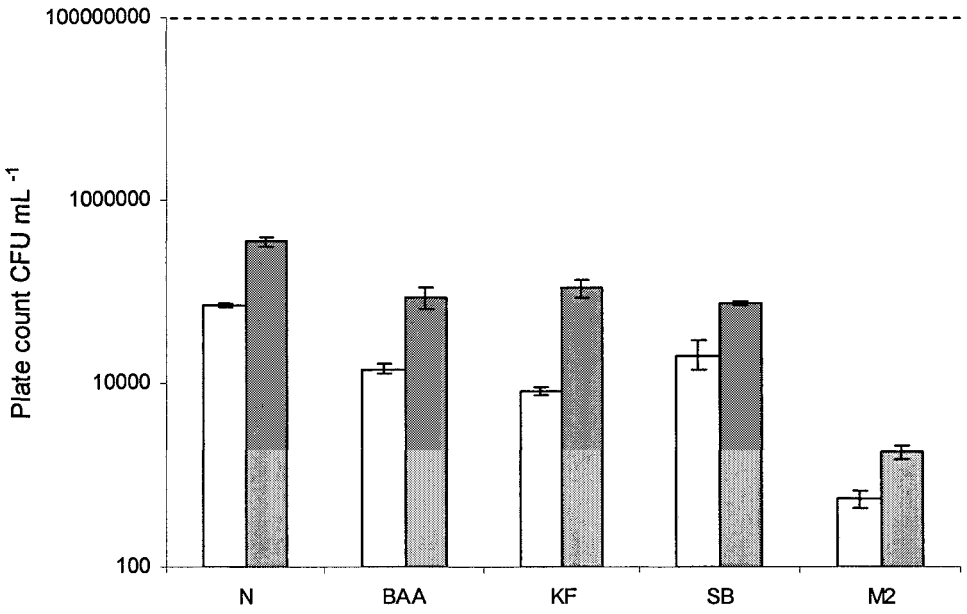


Figure 4.7 Effect of enumeration conditions on *E. faecalis* NCTC775 after exposure to high temperature of 52°C for 90 minutes enumerated on non-selective nutrient agar (N),) and various selective media, namely bile aesculin agar (BAA), KF streptococcus agar (KF), Slanetz and Bartley agar (SB), or MacConkey agar number 2 (M2), incubated either aerobically in unsupplemented medium (unshaded bars), or under ROS-neutralised conditions i.e. medium supplemented with 0.05% sodium pyruvate and incubated in an anaerobic jar (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 4.8a-c depicts results obtained for three *E. faecalis* strains, i.e. a further laboratory strain and two Indian environmental isolates for a similar experiment to that for *E. faecalis* NCTC775 (Fig. 4.7) but using a narrower range of growth media. Fig. 4.8a shows data for *E. faecalis* ATCC35550 exposed to a high temperature of 52°C for 90 minutes, whereas Fig. 4.8b and Fig. 4.8c represents equivalent data for *E. faecalis* PTO1 and *E. faecalis* PTO2 exposed for 90 minutes at 54°C, as preliminary experiments for 90 minutes at 52°C showed minimal decreases in count compared to the initial inoculum. Overall the broad trends in results were similar to those obtained for *E. faecalis* NCTC775 (Fig. 4.7). The non-selective nutrient agar gave higher counts under both enumeration conditions compared with the selective media. Describing the results for strain ATCC35550 in Fig 4.8a, the ROS-neutralised conditions gave a higher count for all three media when compared with standard aerobic conditions. The increases in count under ROS-neutralised conditions were almost 3-fold for nutrient agar and Slanetz & Bartley agar, while KF streptococcus agar gave a 6-fold higher count. A somewhat lesser ROS-neutralised effect was seen for selective media in the case of ATCC35550 Fig. 4.8a) as compared to *E. faecalis* NCTC775 (Fig. 4.7). The two environmental isolates in Fig 4.8b-c also depicted broadly similar results, with isolate PTO1 (Fig. 4.8b) under ROS-neutralised conditions showing increases in count across all growth media with nutrient agar giving more than 2.5-fold increase in count, KF streptococcus giving 18-fold and Slanetz & Bartley agar giving 8-fold higher count compared with aerobic incubation conditions, while strain PTO2 (Fig. 4.8c) showing increases of 2-fold to 3-fold across all growth media.

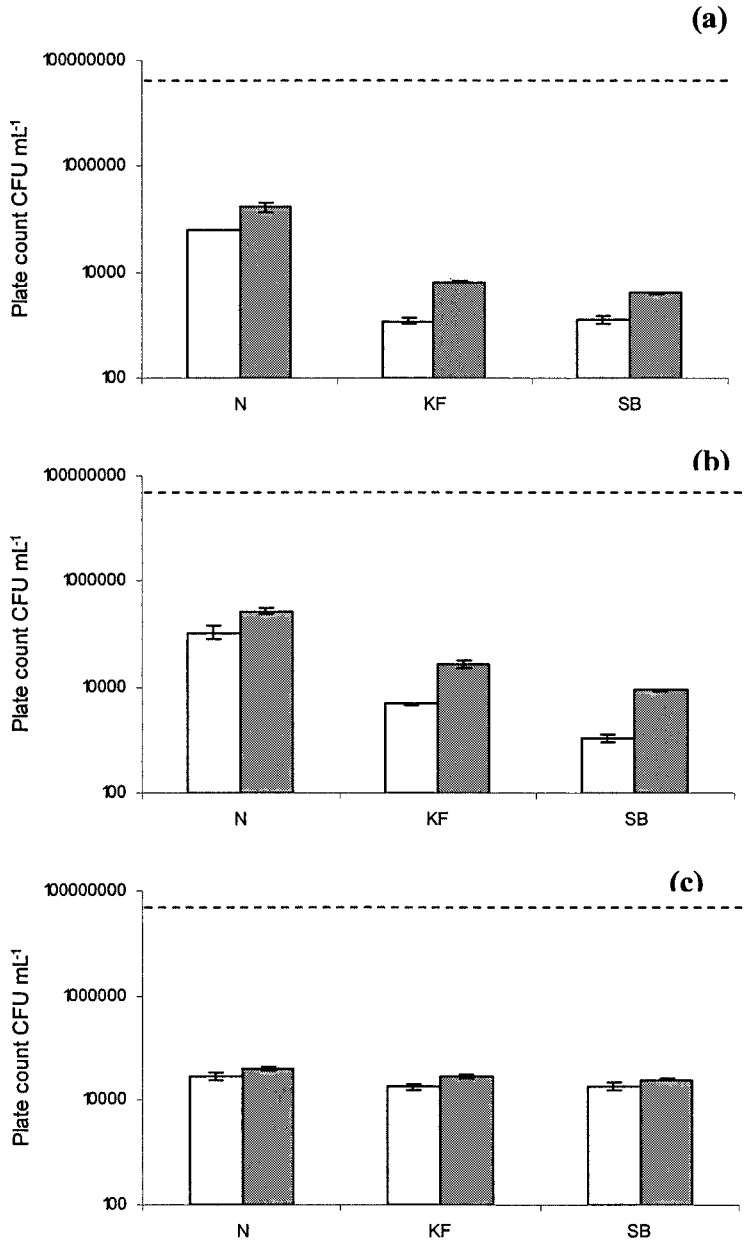


Figure 4.8 Effect of enumeration conditions on three strains of *E. faecalis* after exposure to high temperatures (a) *E. faecalis* ATCC35550 after 90 minutes at 52°C, (b) *E. faecalis* PTO1 after 90 minutes at 54°C, (c) *E. faecalis* PTO2 after 90 minutes at 54°C; enumerated on non-selective nutrient agar (N), KF streptococcus agar (KF), or Slanetz and Bartley agar (SB), incubated either aerobically in unsupplemented medium (unshaded bars), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

4.3.3 Effects of sodium hypochlorite on enumeration of faecal indicator bacteria

***Escherichia coli:* effects of sodium hypochlorite** Preliminary experiments were carried out to determine the exposure time required to cause sub-lethal injury for each of two chlorine solutions: Table 4.3 shows the mean plate counts for *E. coli* NCTC8912 suspended in each solution, i.e. control solution and sodium hypochlorite containing 0.3 $\mu g\ mL^{-1}$ and 0.6 $\mu g\ mL^{-1}$ free chlorine for up to 120 minutes. The enumeration was carried out using nutrient agar under the following four growth conditions, i.e. (i) aerobic conditions with unsupplemented medium, (ii) peroxide-neutralised conditions (aerobic conditions with medium supplemented with 0.05% w/v sodium pyruvate), (iii) anaerobic conditions with unsupplemented medium (in an anaerobic jar), and (iv) ROS-neutralised conditions (anaerobic conditions with medium supplemented with 0.05% w/v sodium pyruvate). The results clearly indicated that 0.6 $\mu g\ mL^{-1}$ free chlorine caused a rapid decrease in cell counts, especially when enumerated under aerobic conditions, reaching undetectable levels within 60 min; however, the counts obtained under conditions designed to neutralize ROS were higher, with anaerobic conditions showing an increase in count followed by peroxide-neutralization, and ROS-neutralisation giving a further enhancement in counts and dropping below the detection limit only after 120 min. A similar overall pattern was seen using dilute hypochlorite solution at 0.3 $\mu g\ mL^{-1}$ free chlorine, though the decreases observed at each time point were lower than the corresponding values for 0.6 $\mu g\ mL^{-1}$. Conversely, incubation in the chlorine-free quarter-strength Ringer's solution for up to 120 min caused no substantial change in cell count, with no evidence of a substantial difference in count under any of the four different enumeration conditions.

Table 4.3 Effects of 12% w/v sodium hypochlorite on the enumeration of *E. coli* NCTC8912 using nutrient agar Mean counts are shown (upper and lower 95% confidence limits in brackets below each mean value) for *E. coli* exposed to free chlorine, incubated either aerobically (unsupplemented medium), or aerobically with supplementation of the medium with 0.05% w/v sodium pyruvate (peroxide-neutralised conditions), anaerobically (anaerobic jar), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised conditions).

		Mean count (CFU mL^{-1})			
Sodium hypochlorite	**Time (min)**	**Aerobic**	**peroxide-neutralised**	**Anaerobic**	**ROS-neutralised**
0 (control in dark at 25°C)	0	5.0×10^7 (4.2×10^7- 6.1×10^7)	5.6×10^7 (5.0×10^7-6.2×10^7)	5.1×10^7 (4.2×10^7- 6.1×10^7)	5.6×10^7 (4.7×10^7-6.6×10^7)
	120	4.2×10^7 (3.6×10^7-4.8×10^7)	4.5×10^7 (3.9×10^7-5.1×10^7)	4.3×10^7 (3.7×10^7-5.0×10^7)	4.5×10^7 (4.2×10^7-4.9×10^7)
0.6 μg mL^{-1}	15	6.2×10^4 (5.6×10^4-6.8×10^4)	2.5×10^5 (1.9×10^5-3.2×10^5)	1.3×10^5 (7.5×10^5-2.1×10^4)	4.6×10^5 (3.7×10^5-5.6×10^5)
	30	3.7×10^3 (3.1×10^3-4.4×10^3)	8.0×10^3 (7.4×10^3-8.6×10^3)	1.2×10^4 (7.1×10^3-2.0×10^4)	4.3×10^4 (3.7×10^4-5.0×10^4)
	60	<50	<50	<50	5.5×10^2 (1.7×10^2-1.8×10^3)
	120	<50	<50	<50	<50
0.3 μg mL^{-1}	15	5.2×10^6 (4.4×10^6-6.3×10^6)	6.1×10^6 (5.2×10^6-7.1×10^6)	6.0×10^6 (4.7×10^6-7.2×10^6)	6.7×10^6 (5.8×10^6-7.8×10^6)
	30	4.7×10^4 (4.1×10^4-5.3×10^4)	2.7×10^5 (1.9×10^5-3.9×10^5)	1.2×10^5 (6.6×10^4-2.0×10^5)	2.9×10^5 (2.3×10^5-3.6×10^5)
	60	5.4×10^3 (4.8×10^3 -6.1×10^3)	1.6×10^4 (1.1×10^4-2.4×10^4)	1.2×10^4 (7.1×10^3-2.0×10^4)	3.4×10^4 (2.8×10^4-4.1×10^4)
	120	5.3×10^2 (3.0×10^2-9.6×10^2)	2.0×10^3 (1.3×10^3-3.2×10^3)	3.6×10^3 (3.0×10^3-4.2×10^3)	5.4×10^3 (4.5×10^3-6.4×10^3)

On the basis of the results shown in Table 4.3, an exposure time of 30 min to sodium hypochlorite at 0.6 μg mL^{-1} free chlorine was selected for further experimentation using different growth media. Fig. 4.9 shows plate counts for chlorine-injured *E .coli* NCTC8912 obtained using nutrient agar (non-selective medium), alongside a range of selective media, enumerated under three sets of conditions, i.e. in standard aerobic medium, peroxide-neutralised medium and ROS-neutralised conditions. There was a substantial decrease in counts (1500-450000 fold) compared to the initial inoculum, with the reduction depending upon the growth medium and enumeration conditions. Comparing the various selective media under standard aerobic conditions, MacConkey agar and mFC-R agar gave a similar count to nutrient agar, while mLSA gave the lowest aerobic count and m-Endo medium gave intermediate values. In peroxide-neutralised conditions (pyruvate-supplemented medium under aerobic conditions) the values for each medium were somewhat higher than the corresponding aerobic counts, with almost the same trend in terms of the relative efficacy of each medium. Under ROS-neutralised conditions, all media showed a further increase in colony count, with nutrient agar giving a value around 5-fold higher, while the selective media gave more variable increases. Thus for each medium, the count obtained under ROS-neutralised conditions was notably higher than the corresponding aerobic count. Overall, MacConkey agar and mFC-R medium gave similar counts to those of nutrient agar.

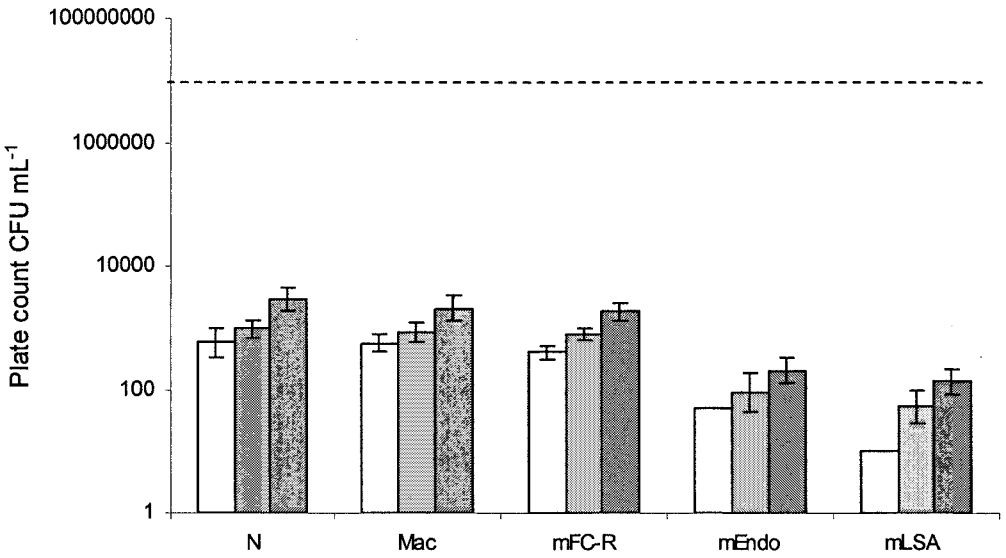


Fig. 4.9 Effects of enumeration conditions on chlorine-stressed *Escherichia coli* NCTC8912 Mean plate count (CFU mL^{-1}), following 30 min chlorine injury by 0.6 μg ml^{-1} of free chlorine solution, enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, and m-FC agar without rosolic acid (mFC-R), incubated either aerobically in unsupplemented medium (unshaded bars), or peroxide-neutralised with aerobic incubation in medium supplemented with 0.05% w/v sodium pyruvate (light-shaded), or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 4.10a-c provides data for a similar experiment carried out for 3 other strains of *E. coli* using the same media but only two incubation conditions, i.e. standard aerobic conditions and ROS-neutralizing conditions. Fig. 4.10a shows data for TN675 suspended 30 minutes in 0.6 $\mu g\ mL^{-1}$ of free chlorine, while Fig. 4.10b and Fig. 4.10c shows equivalent results for the two Indian isolates, PUCC061 and PUCC113, suspended for 60 min in same chlorine solution (preliminary results showed that 30 min exposure resulted in minimal change in count of these two environmental isolates). Overall, while the broad trends shows in Fig. 4.10a-c are similar to those observed with *E. coli* NCTC8912 (Fig. 4.9) the differences between the various media and growth conditions are proportionately smaller than for *E. coli* NCTC8912. Thus, non-selective nutrient agar under ROS-neutralised conditions gave around 5-fold (environmental isolates) and 6-fold (TN675) higher counts than under aerobic conditions. In comparing the different selective media, MacConkey agar proved to be least inhibitory and mLSA the most inhibitory in all cases.

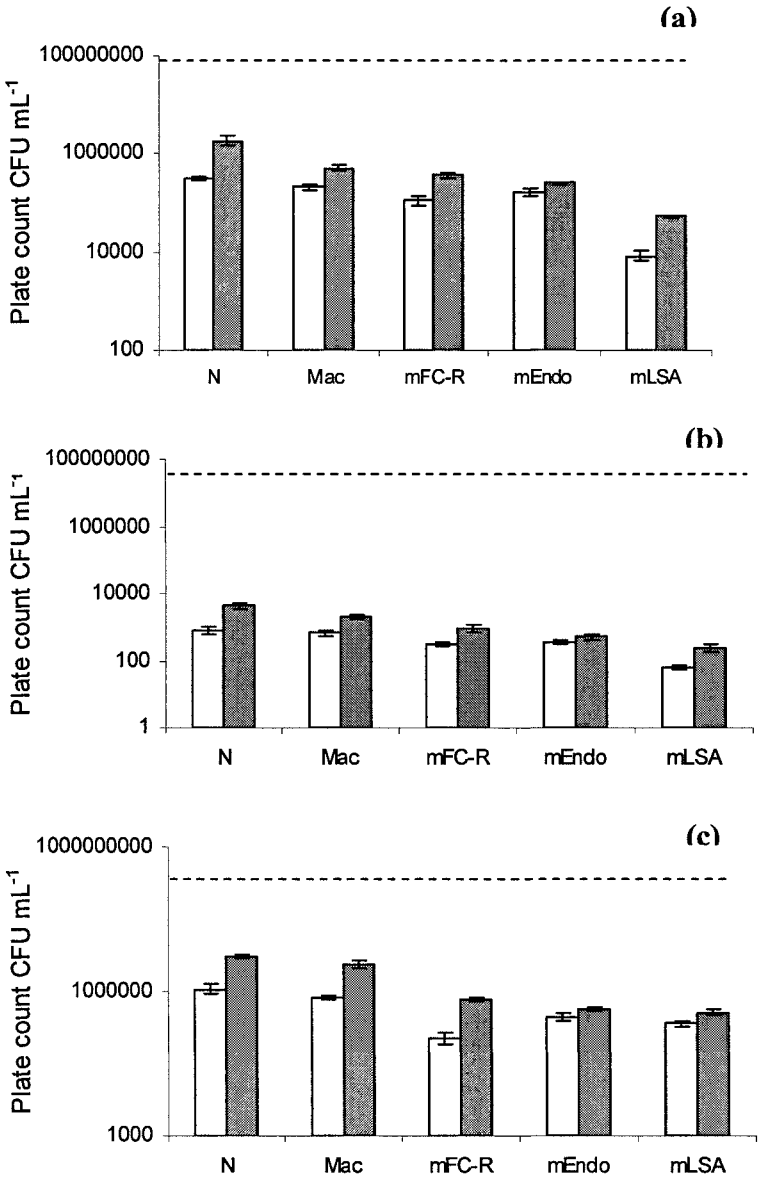


Fig. 4.10 Effects of enumeration conditions on three different strains of *Escherichia coli* after chlorine injury Mean plate count (CFU mL^{-1}) (a) *E. coli* TN675 after 30 minutes; (b) *E. coli* PUCC061 after 60 minutes; (c) *E. coli* PUCC113 after 60 minutes chlorine injury by 0.6 μg ml^{-1} of free chlorine enumerated on non selective nutrient agar (N), and selective, MacConkey agar (Mac), m-Faecal Coliform agar without rosolic acid (mFC-R), m-Endo (E) and m-lauryl sulphate agar (mLSA),and incubating either, under aerobic in unsupplemented medium (unshaded bars), or ROS-neutralised conditions (dark-shaded bars). Initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

***Enterococcus faecalis:* effects of sodium hypochlorite** Plate counts are shown in Fig. 4.11 for *E. faecalis* NCTC775 suspended in sodium hypochlorite solution at a free chlorine level of 0.9 $\mu g\ mL^{-1}$ for 30 minutes, enumerated on nutrient agar a non-selective medium and four selective media, i.e. bile aesculin agar, KF streptococcus agar, Slanetz & Bartley agar and MacConkey agar number 2, under standard aerobic (unsupplemented) conditions and ROS-neutralised conditions. It is evident from the results that there was a substantial decrease in counts compared to the initial inoculum with the reduction varying from 150-fold to 100000-fold, depending both upon growth medium and enumeration conditions. Comparing the selective media incubated under aerobic conditions with non selective nutrient agar, KF streptococcus agar gave almost 10-fold lower count than nutrient agar, while a 20-fold lower count was seen for Slanetz & Bartley agar, whereas MacConkey agar number 2 gave the lowest count of 150-fold lower than nutrient agar. Under ROS-neutralised conditions enhancements in counts were seen for all media, with nutrient agar showing an increase of 4-fold, with the greatest increase of 26-fold seen for KF streptococcus agar, reaching a value similar to that of nutrient agar while Slanetz & Bartley agar and MacConkey agar number 2 showed far smaller increases. In general both non-selective and selective media showed enhancements in counts under ROS-neutralised conditions for *E. faecalis* NCTC775 when compared with its aerobic counterpart, though not always to the same extent.

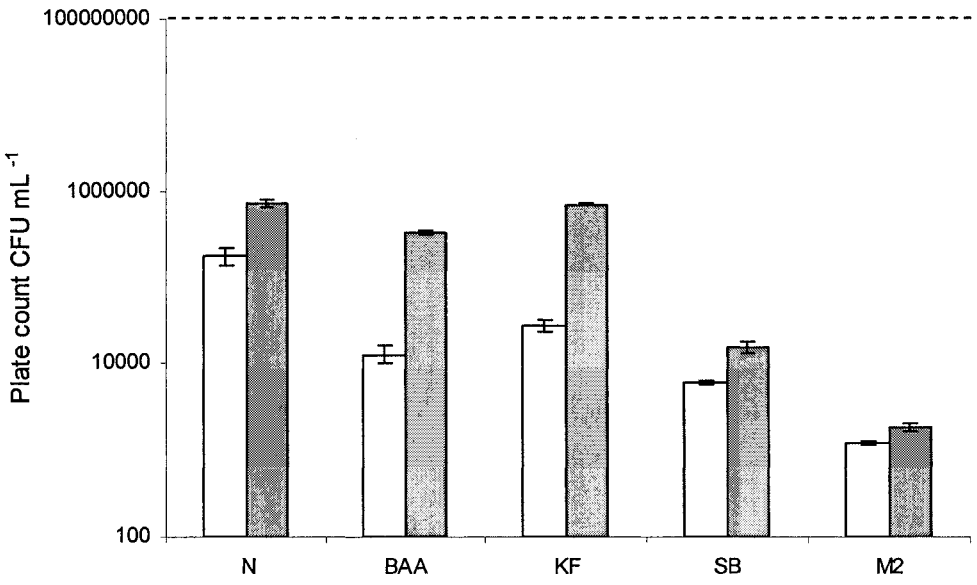


Fig. 4.11 Effect of enumeration conditions on *E. faecalis* NCTC775 after chlorine injury Mean plate count (CFU mL^{-1}) for *E. faecalis* NCTC775 after exposure to free chlorine solution of 0.9 $\mu g\ mL^{-1}$ for 30 minutes, enumerated on non-selective nutrient agar (N), and various selective media, namely bile aesculin agar (BAA), KF streptococcus agar (KF), Slanetz and Bartley agar (SB), or MacConkey agar no.2 (M2), incubated either aerobically in unsupplemented medium (unshaded bars), or under ROS-neutralised conditions i.e. medium supplemented with 0.05% sodium pyruvate and incubated in an anaerobic jar (dark-shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 4.12a-c provides data for experiments conducted using three other strains of *E. faecalis* after chlorine injury, under similar enumeration conditions but using a narrower range of growth media. Fig. 4.12a depicts results for *E. faecalis* ATCC35550 exposed for 30 minutes, while Fig. 4.12b and Fig. 4.12c represents equivalent data for *E. faecalis* PTO1 and *E. faecalis* PTO2, exposed for 60 minutes in the same chlorine solution (0.9 $\mu g\ mL^{-1}$ of free chlorine). The exposure time was increased to 60 minutes as preliminary results for 30 minutes exposure showed minimal decrease in cell count for these two environmental isolates. The broad trend in results is similar to that obtained for *E. faecalis* NCTC775 (Fig. 4.11), but with smaller differences in count between the various growth conditions and media. The non-selective nutrient agar showed a higher count compared to the two selective media. An enhancement in count of almost 3-fold under ROS-neutralised conditions was obtained for nutrient agar when compared to aerobic conditions. The selective media also showed increases in count under ROS-neutralised conditions but these increases were more variable.

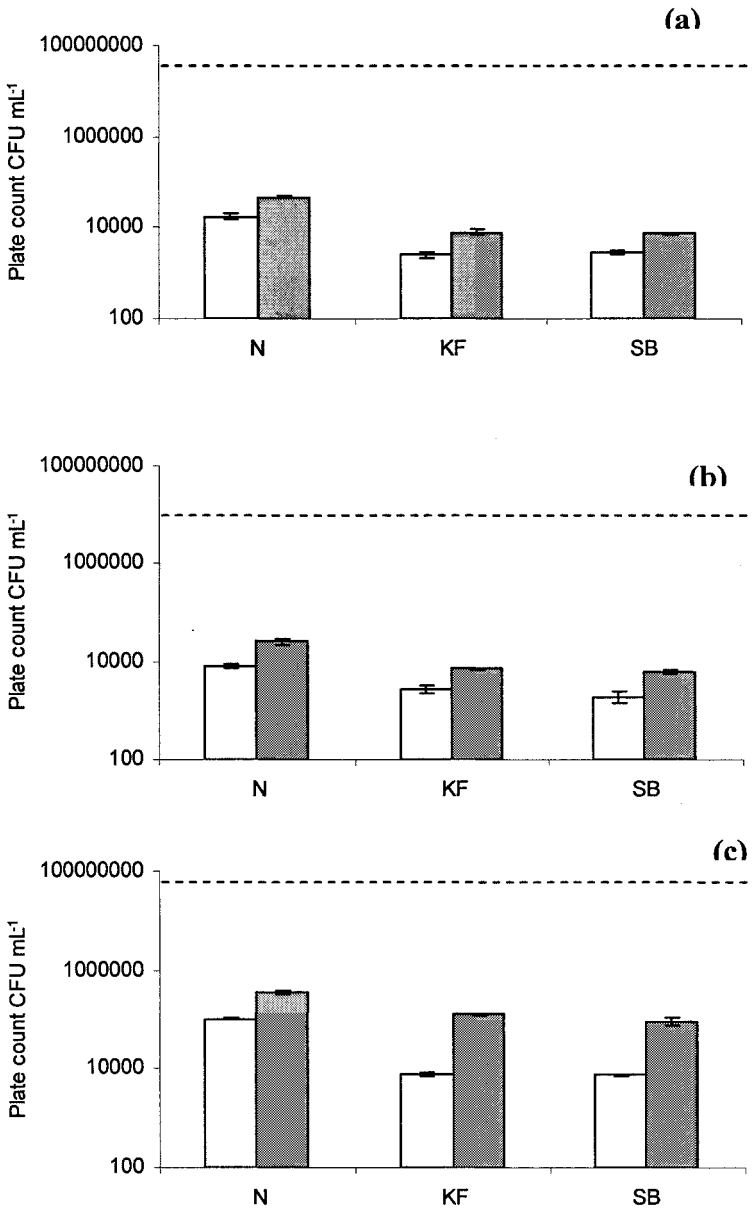


Fig. 4.12 Effect of enumeration conditions on three different strains of *E. faecalis* after free chlorine exposure of 0.9 μg mL^{-1} (a) *E. faecalis* ATCC35550 for 30 minutes, (b) *E. faecalis* PTO1 0.9 μg mL^{-1} for 60 minutes, (c) *E. faecalis* PTO2 for 60 minutes; enumerated on non-selective nutrient agar (N), KF streptococcus agar (KF), or Slanetz and Bartley agar (SB), incubated either aerobically in unsupplemented medium (unshaded bars), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

4.3.4 Effects of low pH on the enumeration of faecal indicator bacteria

***Escherichia coli:* effects of low pH** Preliminary experiments were carried out to optimise the exposure time required to give an indication of sub-lethal injury induced by low pH. Table 4.4 shows the mean plate counts for *E. coli* NCTC8912 suspended in each solution, i.e. pH=2 (low pH) and 7 (control) for up to 24 hours, with enumeration on nutrient agar under four sets of conditions, i.e. (i) aerobic conditions with unsupplemented medium, (ii) peroxide-neutralised conditions, (iii) anaerobic conditions (anaerobic jar) with unsupplemented medium and (iv) ROS-neutralised conditions. The results show that an acidic pH value of 2 caused a rapid decrease in cell counts under all four sets of enumeration conditions, reaching levels which were undetectable after 6 h. At 3 h a decrease in overall initial count was seen at pH 2, while the extent of the decrease did not depend upon enumeration conditions as it gave similar count under all four sets of growth conditions. Conversely, incubation in the quarter-strength Ringer's solution at pH 7 for 24 h caused no substantial change in cell count, with no evidence of a significant difference in count and therefore no sign of sub-lethal injury.

Table 4.4 Effects of low pH on the enumeration of *E. coli* NCTC8912 using nutrient agar
Mean counts are shown (upper and lower 95% confidence limits in brackets below each mean value) for *E. coli* exposed to low pH, incubated either aerobically (unsupplemented medium), or aerobically with supplementation of the medium with 0.05% w/v sodium pyruvate (peroxide-neutralised conditions), anaerobically (anaerobic jar), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised conditions).

		Mean count (CFU mL^{-1})			
pH	**Time (h)**	**Aerobic**	**Peroxide-neutralised**	**Anaerobic**	**ROS-neutralised**
pH =7 (control in dark at 25°C)	0	3.0×10^7 (2.4×10^7-3.7×10^7)	3.6×10^7 (2.8×10^7-4.7×10^7)	3.2×10^7 (2.6×10^7-4.0×10^7)	3.8×10^7 (3.0×10^7-5.0×10^7)
	24	1.7×10^7 (7.6×10^7-3.6×10^7)	1.8×10^7 (1.1×10^7-3.1×10^7)	1.7×10^7 (1.7×10^7)	2.0×10^7 (1.5×10^7-2.8×10^7)
pH =2	3	1.5×10^3 (8.1×10^2-3.0×10^3)	2.0×10^3 (1.0×10^3-3.7×10^3)	1.9×10^3 (1.2×10^3-3.6×10^3)	2.0×10^3 (2.0×10^3)
	6	<50	<50	<50	<50
	24	<50	<50	<50	<50

On the basis of the results shown in Table 4.4, an exposure time of 3 h to pH 2 was selected for further experimentation. Fig. 4.13 shows plate counts for *E. coli* NCTC8912 enumerated using nutrient agar (non selective medium) alongside a range of selective media, (i) in standard medium under aerobic conditions, (ii) in peroxide-neutralised medium, and (iii) under ROS-neutralised conditions. There was a considerable decrease in counts compared to the initial inoculum, with the reduction in count obtained ranging between 25-fold to 80-fold, depending on the medium used. In contrast to the previous stressors tested, the diminution in count at low pH was broadly similar on all growth media and under all enumeration conditions. Thus plate counts on non-selective medium and on the various selective media under standard aerobic conditions, or pyruvate-neutralising conditions, or ROS-neutralizing conditions were similar with minimal differences seen across the various enumeration conditions.

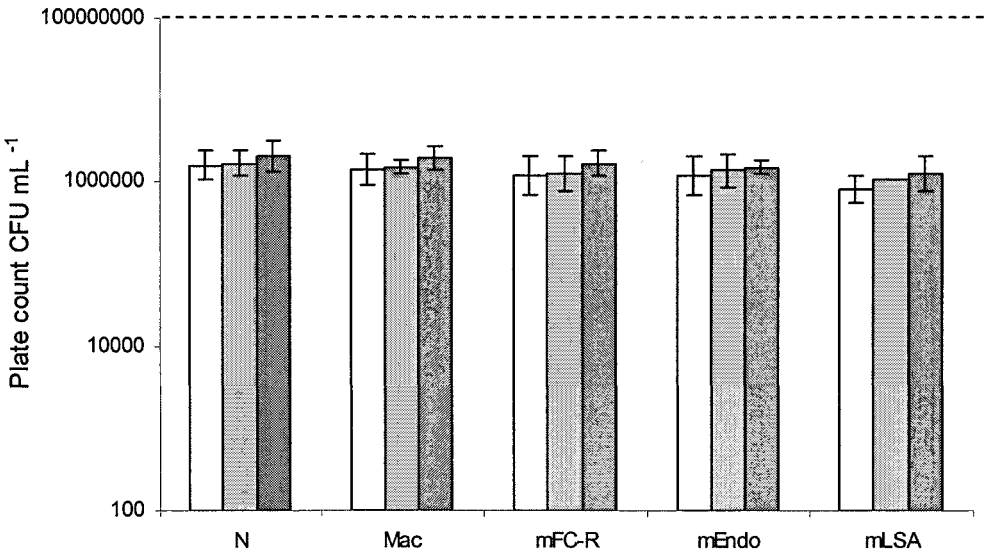


Fig. 4.13 Effects of enumeration conditions on *Escherichia coli* NCTC8912 after exposure to low pH Mean plate count (CFU ml^{-1}) following exposure to pH 2 for 3 h, enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), m-lauryl sulphate agar (mLSA), mEndo medium, and m-FC agar without rosolic acid (mFC-R), incubated either aerobically in unsupplemented medium (unshaded bars), or peroxide-neutralised with aerobic incubation in medium supplemented with 0.05% w/v sodium pyruvate (light-shaded), or anaerobically (anaerobic jar) with supplementation of the medium with 0.05% w/v sodium pyruvate (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Fig. 4.14a-c illustrates results for plate counts for a similar experiment carried out for 3 other strains of *E. coli* using the same selective media but comparing only two sets of growth conditions, i.e. (i) standard aerobic conditions and (ii) ROS-neutralised conditions. Fig. 4.14a provides data for *E. coli* TN675 a laboratory strain while Fig. 4.14b gives results for *E. coli* PUCC061 and Fig. 4.14c for *E. coli* PUCC113, two environmental isolates. All three strains were exposed for 45 min to water of low pH. Overall, the broad trends shown in Fig. 4.14a-c are similar to those observed with *E. coli* NCTC8912 (Fig. 4.13), i.e. minimal differences between the various growth media and sets of enumeration conditions.

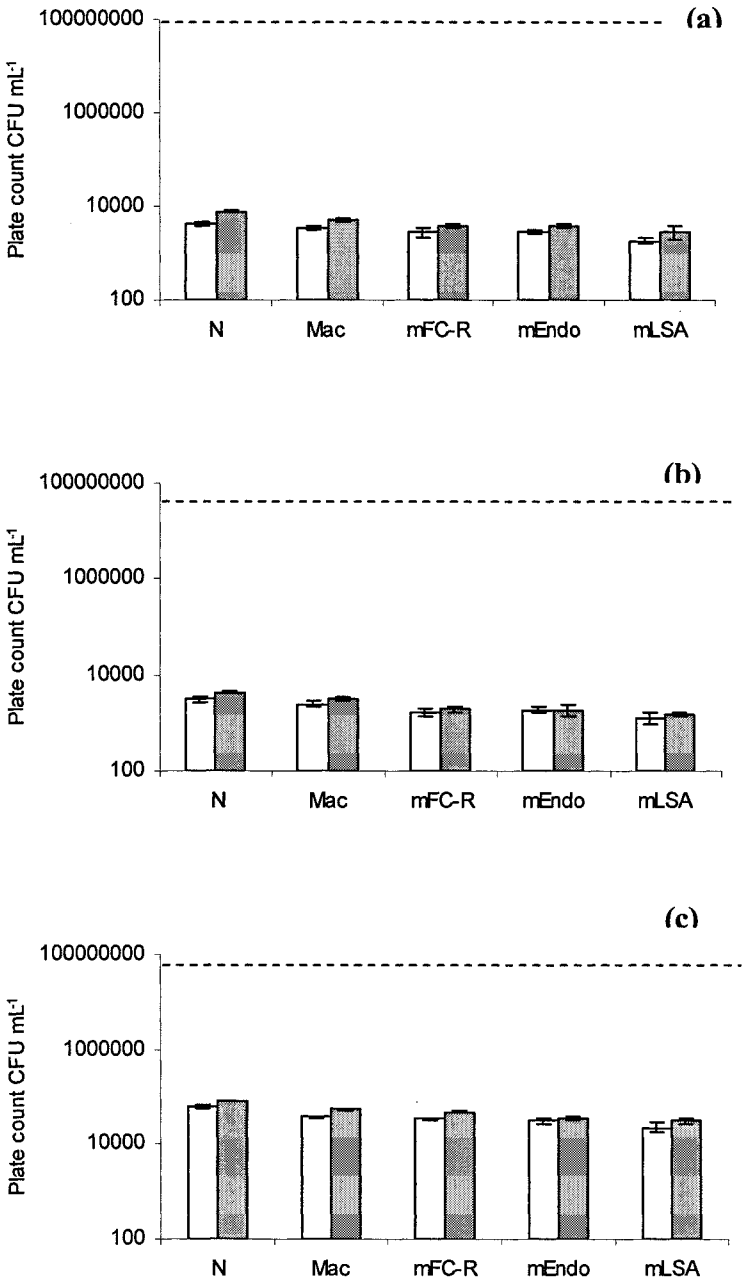


Fig. 4.14 Effects of enumeration conditions on different strains of *Escherichia coli* after exposure to low pH of 2 Mean plate count (CFU ml^{-1}) (a) *E. coli* TN675 after 45 minutes; (b) *E. coli* PUCC061 after 45 minutes; (c) *E. coli* PUCC113 after 45 minutes, enumerated on non selective nutrient agar (N), and selective, MacConkey agar (Mac), m-Faecal Coliform agar without rosolic acid (F-R), m-Endo (E) and m-lauryl sulphate agar (L),and incubating either, under aerobic conditions using unsupplemented medium (unshaded bars), or ROS-neutralised conditions (dark-shaded bars). Initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

***Enterococcus faecalis:* effects of low pH** Fig. 4.15 shows results obtained for *E. faecalis* NCTC775 after suspension in water of low pH for 30 minutes, enumerated on non-selective nutrient agar and different selective media, i.e. bile aesculin agar, KF Streptococcus agar, Slanetz and Bartley agar and MacConkey agar number 2 under standard aerobic and ROS-neutralised conditions. There was a reduction in count compared to the initial inoculum with 19000-fold to 30000-fold decreases in count seen after exposure to acidic pH. The decrease in count was similar on different growth media used irrespective whether they were non-selective or selective. Furthermore, this reduction was not dependent upon enumeration conditions, whether standard aerobic or ROS-neutralised.

Fig 4.16 a-c displays results for an equivalent experiment conducted using three other strains of *E. faecalis*, i.e. a laboratory strain ATCC35550 (Fig. 4.16a), two environmental isolates PTO1 (Fig 4.16b) and PTO2 (Fig. 4.16c) suspended in low pH solution for 30 minutes but using a narrower range of growth media, i.e. non-selective nutrient agar, selective KF Streptococcus agar and Slanetz and Bartley agar under same type of enumeration conditions. Overall the results obtained in Fig. 4.16 a-c after exposure to low pH are similar to those shown for strain NCTC775 in Fig. 4.15 with, firstly a substantial decrease in cell count compared with the initial inocula, secondly the decrease is similar in terms of the cell count on non-selective or selective media and irrespective of whether the plates were incubated aerobically or under ROS-neutralised conditions.

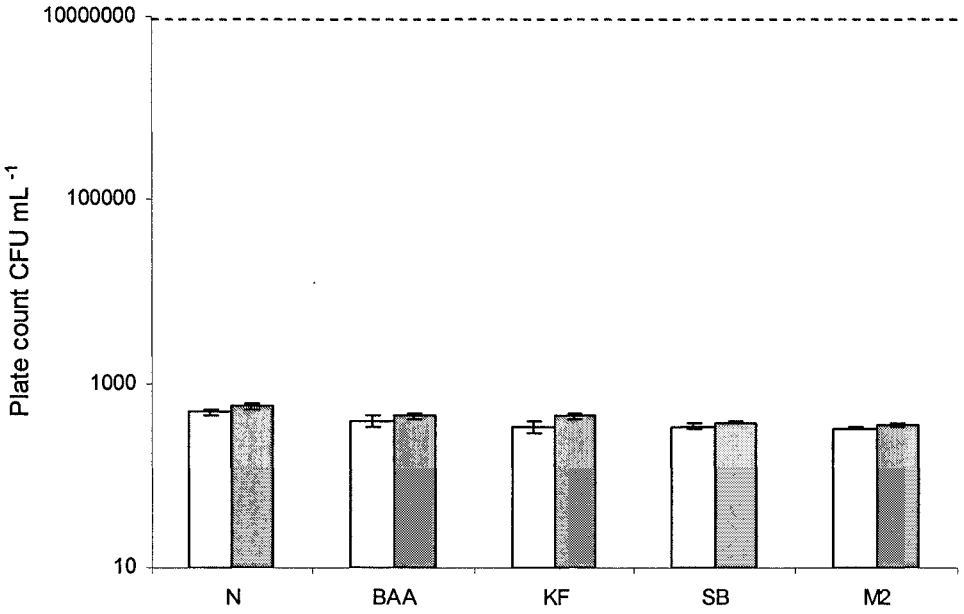


Figure 4.15 Effect of enumeration conditions on *E. faecalis* NCTC775 after exposure to low pH Mean plate count (CFU ml^{-1}) for *E. faecalis* NCTC775 after exposure to low pH of 2 for 30 minutes, enumerated on non-selective nutrient agar (N) and various selective media, namely bile aesculin agar (Bile), KF streptococcus agar (KF), Slanetz and Bartley agar (SB), or MacConkey agar no.2 (M2), incubated either aerobically in unsupplemented medium (unshaded bars), or under ROS-neutralised conditions i.e. medium supplemented with 0.05% sodium pyruvate and incubated in an anaerobic jar (dark-shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

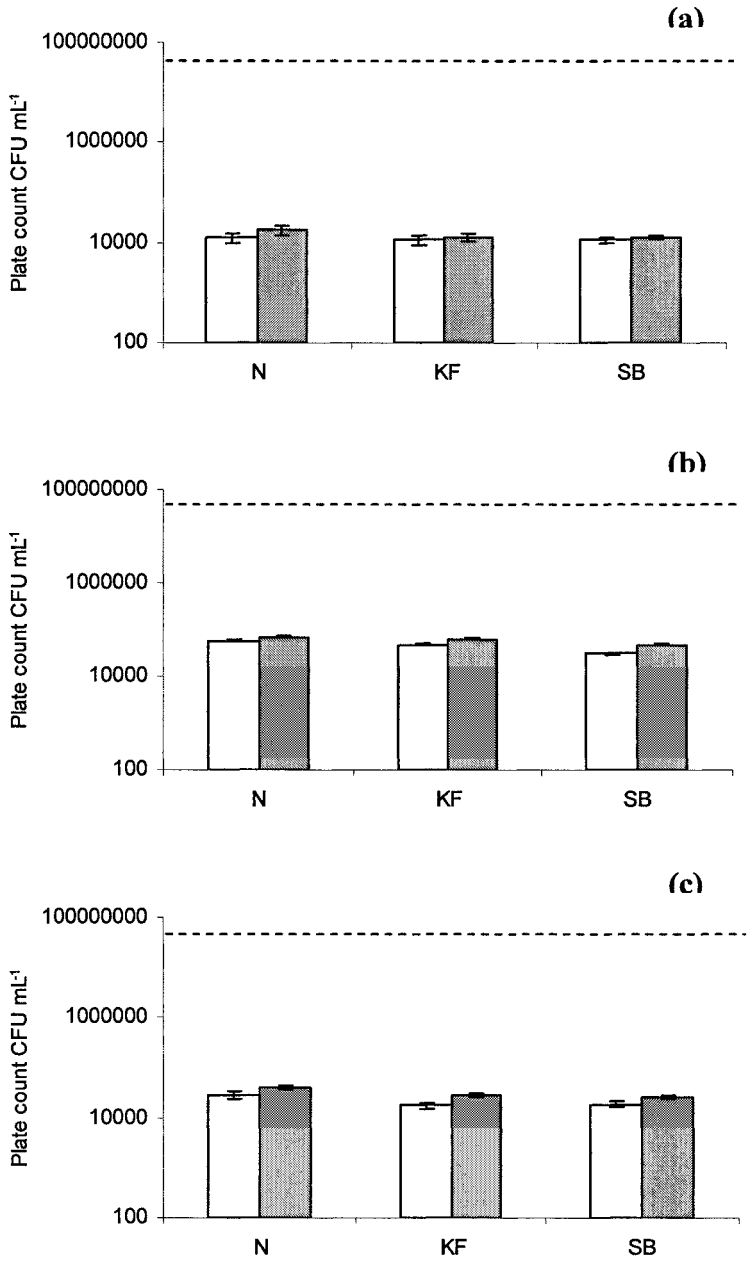


Figure 4.16 Effect of enumeration conditions on three strains of *E. faecalis* after exposure to low pH of 2 (a) *E. faecalis* ATCC35550 after 30 minutes, (b) *E. faecalis* PTO1 after 60 minutes, (c) *E. faecalis* PTO2 after 60 minutes; enumerated on non-selective nutrient agar (N), KF streptococcus agar (KF), or Slanetz and Bartley agar (SB), incubated either aerobically in unsupplemented medium (unshaded bars), or anaerobically with supplementation of the medium with 0.05% w/v sodium pyruvate in an anaerobic jar (ROS-neutralised; dark-shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

4.3.5 Effects of long-term incubation in sterile distilled water on the enumeration of faecal indicator bacteria

***Escherichia coli:* extended incubation in distilled water** Plate counts are shown in a time course graph for *E. coli* NCTC8912 stored in foil-covered glass flask containing sterile distilled water maintained at 25°C and 37°C in Fig. 4.17a-e for five different enumeration media. Timed samples were taken every fifteen days and enumerated on agar plates with and without pyruvate supplementation using non-selective nutrient agar (Fig. 4.17a) and various selective media, namely MacConkey agar (Fig. 4.17b), mFC-R agar (Fig. 4.17c), mEndo agar (Fig. 4.17d) and mLSA (Fig. 4.17e). The plates were incubated at 37°C under standard aerobic conditions for unsupplemented medium and under anaerobic conditions for pyruvate-supplemented medium.

E. coli NCTC8912 maintained at an incubation temperature of 25°C showed a steady reduction in plate count, displaying decreases almost in a straight line, with counts being within detectable limits up to 120 days and undetectable thereafter. The counts obtained at each time interval were almost identical for both sets of enumeration conditions, with negligible variability in count between non-selective nutrient agar and the various selective media. The same strain of *E. coli* maintained at an incubation temperature of 37°C showed a more rapid reduction in count with more than a 10-fold reduction after 15 days. The counts at 37°C reached undetectable levels within 60 days, which was around half of the time taken for the same strain to reach the same point at 25°C. Furthermore, the extent of the decreases were a little more variable than at 25oC,

giving a less linear decline. However, as at 25°C, the differences obtained between aerobic and ROS-neutralised counts across the various growth media were very small.

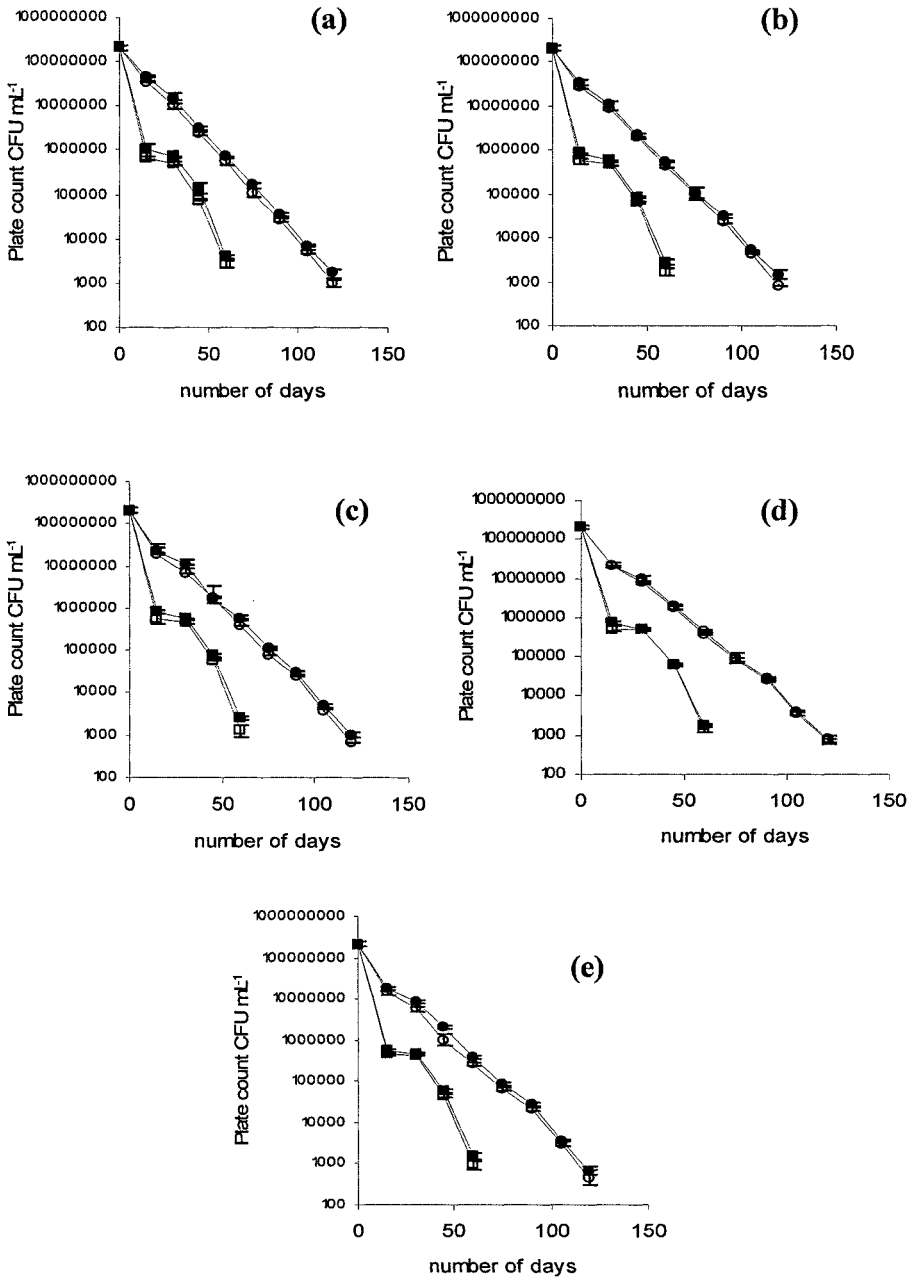


Fig. 4.17 Effects of long-term incubation in sterile distilled water on the enumeration of *E. coli* NCTC8912 at 25°C and 37°C (a) nutrient agar, (b) MacConkey agar, (c) m-Faecal Coliform agar without rosolic acid (mFC-R agar), (d) m-Endo agar, and (e) m-Lauryl sulphate agar (mLSA) incubated either, under aerobic conditions (open symbols), or ROS-neutralised conditions (closed symbols) with incubation temperatures of 25°C (circles) and 37°C (squares). The initial inoculum is represented as start point on Y-axis. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

Similar experiments were conducted using three other strains of *E. coli*, i.e. TN675, PUCC061 and PUCC113. The results for strain TN675 are depicted in Fig. 4.18a-e, displaying a faster decrease in count at 25°C compared to NCTC8912, which was even faster at 37°C. Fig. 4.19a-e and Fig. 4.20a-e shows data for the two environmental isolates of *E. coli,* i.e. PUCC061 and PUCC113. The results for starvation of these two environmental strains depict that there was no ROS-neutralisation effect as seen for other stresses (water stored in brass vessel, exposed to sunlight and high temperatures).

The overall results obtained for all four *E. coli* strains indicate that they can be maintained and preserved in distilled water at a temperature of 25°C for up to three or four months, with a steady decrease in counts during this time. Incubation in sterile distilled water at 25°C maintains the count for longer periods, when compared to 37°C with a high rate of inactivation. Furthermore, a similar count was obtained whether using a non-selective or selective media, with no substantial difference in counts under aerobic or ROS-neutralised conditions.

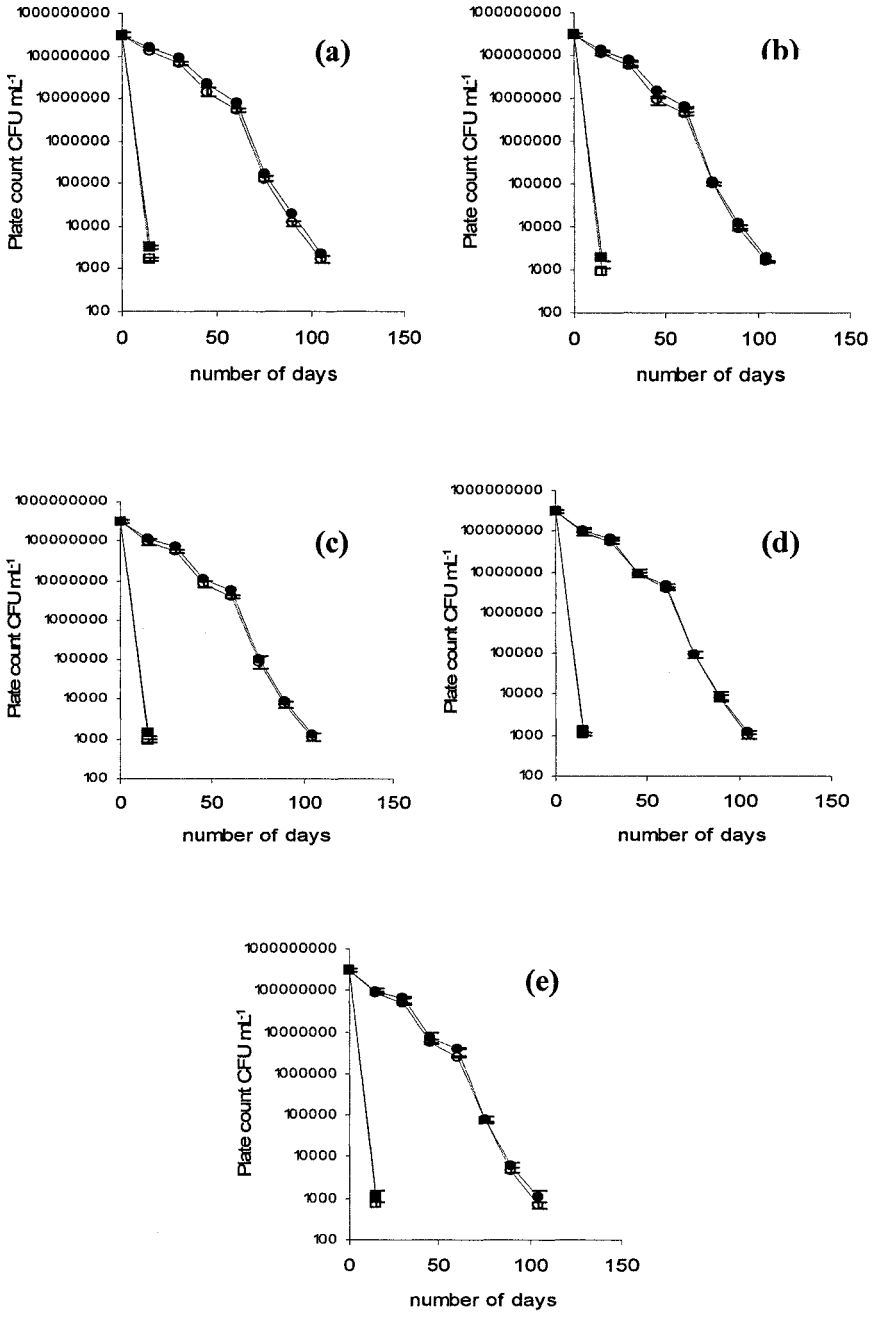


Fig. 4.18 Effects of long-term incubation in sterile distilled water on the enumeration of *E. coli* TN675 at 25°C and 37°C (a) nutrient agar, (b) MacConkey agar, (c) m-Faecal Coliform agar without rosolic acid (mFC-R agar), (d) m-Endo agar, and (e) m-Lauryl sulphate agar (mLSA) incubated either, under aerobic conditions (open symbols), or ROS-neutralised conditions (closed symbols) with incubation temperatures of 25°C (circles) and 37°C (squares). The initial inoculum is represented as start point on Y-axis. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

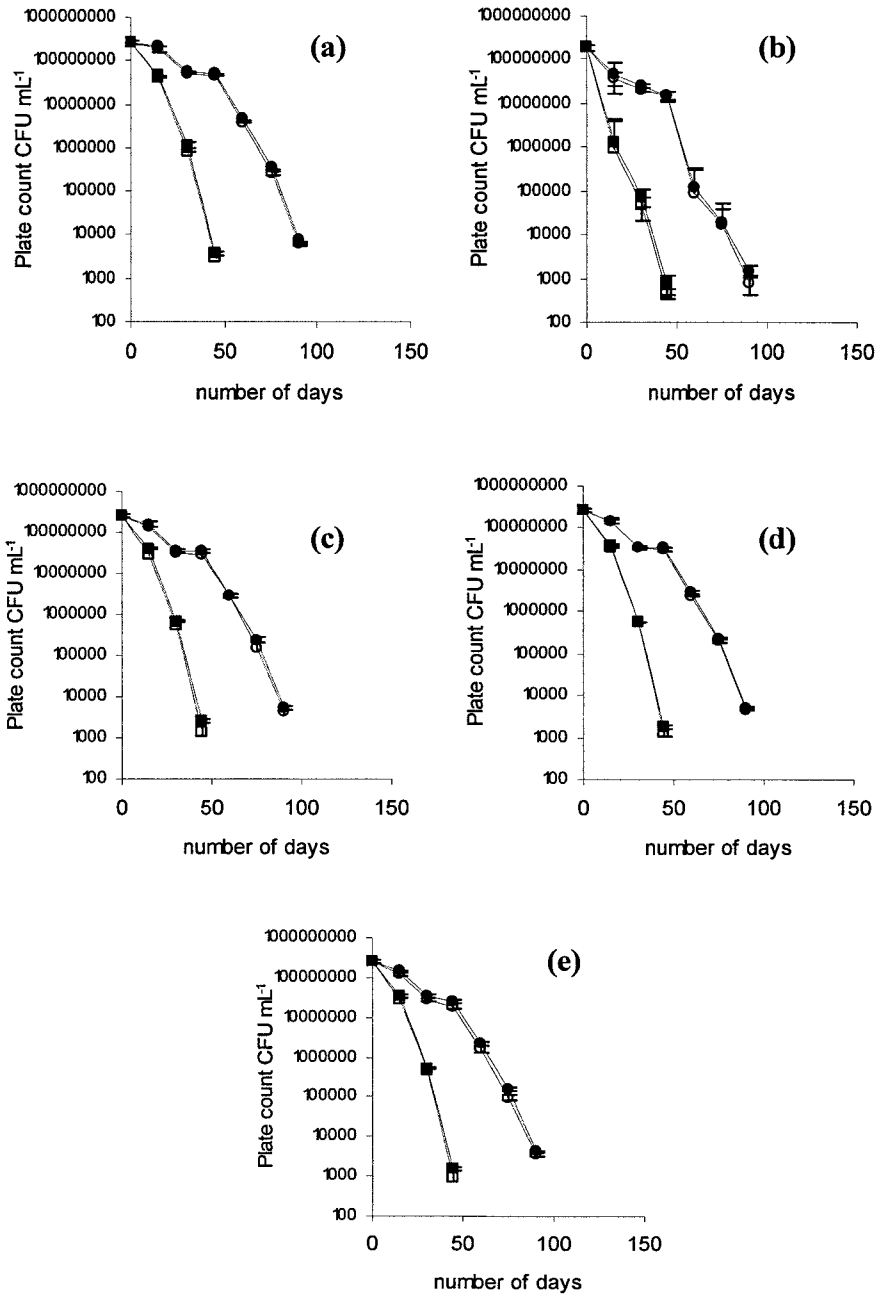


Fig. 4.19 Effects of long-term incubation in sterile distilled water on the enumeration of *E. coli* PUCC0116 at 25°C and 37°C (a) nutrient agar, (b) MacConkey agar, (c) m-Faecal Coliform agar without rosolic acid (mFC-R agar), (d) m-Endo agar, and (e) m-Lauryl sulphate agar (mLSA) incubated either, under aerobic conditions (open symbols), or ROS-neutralised conditions (closed symbols) with incubation temperatures of 25°C (circles) and 37°C (squares). The initial inoculum is represented as start point on Y-axis. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

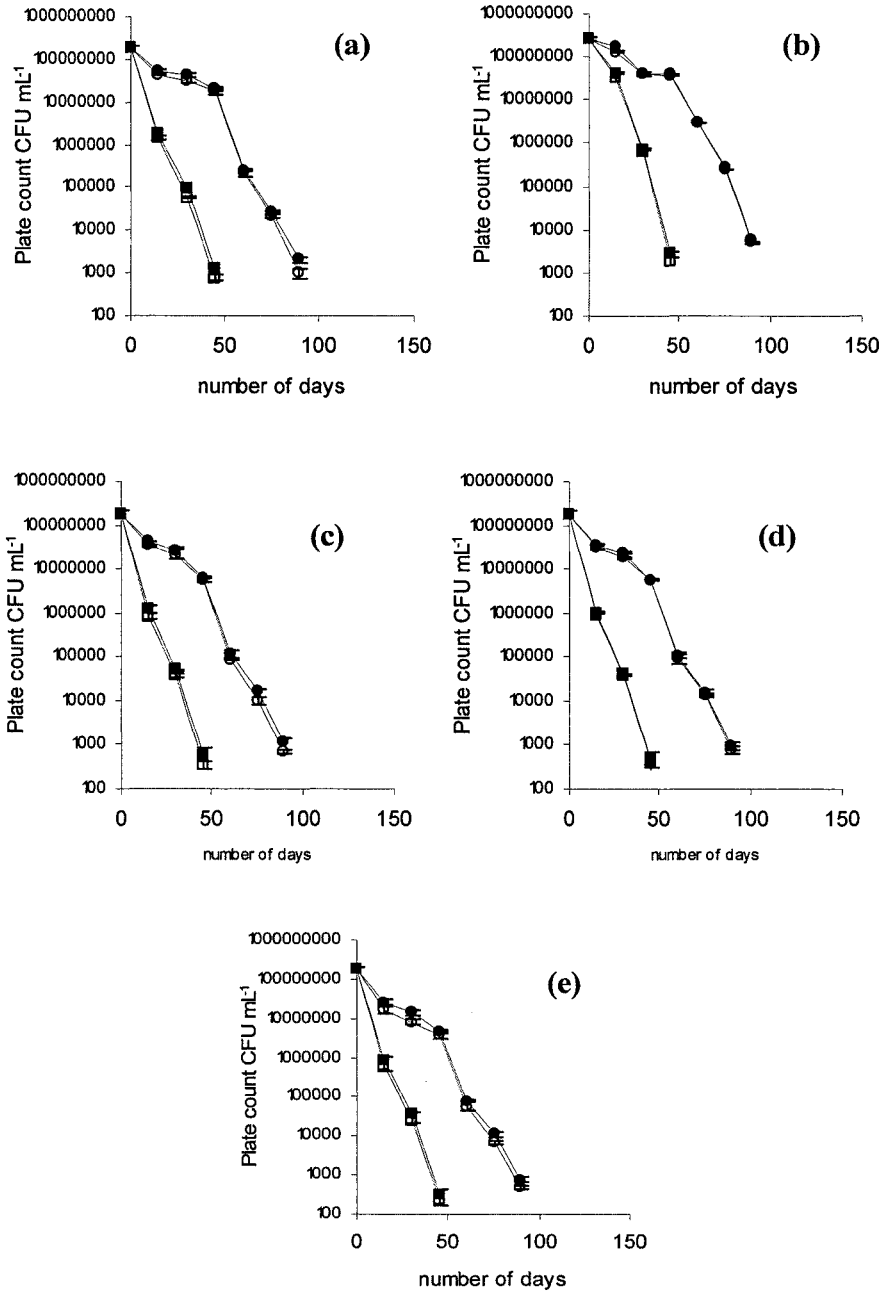


Fig. 4.20 Effects of long-term incubation in sterile distilled water on the enumeration of *E. coli* PUCC113 at 25°C and 37°C (a) nutrient agar, (b) MacConkey agar, (c) m-Faecal Coliform agar without rosolic acid (mFC-R agar), (d) m-Endo agar, and (e) m-Lauryl sulphate agar (mLSA) incubated either, under aerobic conditions (open symbols), or ROS-neutralised conditions (closed symbols) with incubation temperatures of 25°C (circles) and 37°C (squares). The initial inoculum is represented as start point on Y-axis. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

***Enterococcus faecalis:* extended incubation in distilled water**

Plate counts are shown in Fig. 4.21a-c for *E. faecalis* NCTC775 maintained in sterile distilled water in darkness at temperatures of 25°C and 37°C, with enumeration on non-selective nutrient agar and various selective media, i.e. KF streptococcus agar and Slanetz and Bartley agar with incubation at 37°C for 48 h under aerobic conditions (un-supplemented medium) and ROS-neutralised conditions (on pyruvate-supplemented medium in an anaerobic jar). The results for the suspension maintained at 25°C showed a detectable count up to 90 days and for 37°C up to 15 days, thus showing a faster rate of inactivation at the higher temperature, as was seen with *E. coli*. The reduction in count compared to the initial inoculum (6.13×10^7 CFU mL^{-1}) showed minimal differences in count between aerobic conditions and ROS-neutralised conditions. Further the decreases in count were not greatly affected by the growth medium used for enumeration.

Similar experiments were carried out using three other strains of *E. faecalis*, i.e. strain ATCC35550 and the environmental isolates PTO1 and PTO2. The results for ATCC35550 strain are displayed in Fig. 4.22a-c, Fig. 4.23 a-c and Fig. 4.24a-c for the three test media at 25°C and 37°C under aerobic conditions and ROS-neutralised conditions. In broad terms the pattern of results in these three strains of *E. faecalis* are similar to that obtained for strain NCTC775, with similar count on all sets of media and growth conditions at both temperatures, and with cell suspensions stored at 37°C giving a more rapid reduction in count than at 25°C, being undetectable by 30 days at 37°C and with no evidence of any substantial effect of nutrient composition, or ROS-neutralisation.

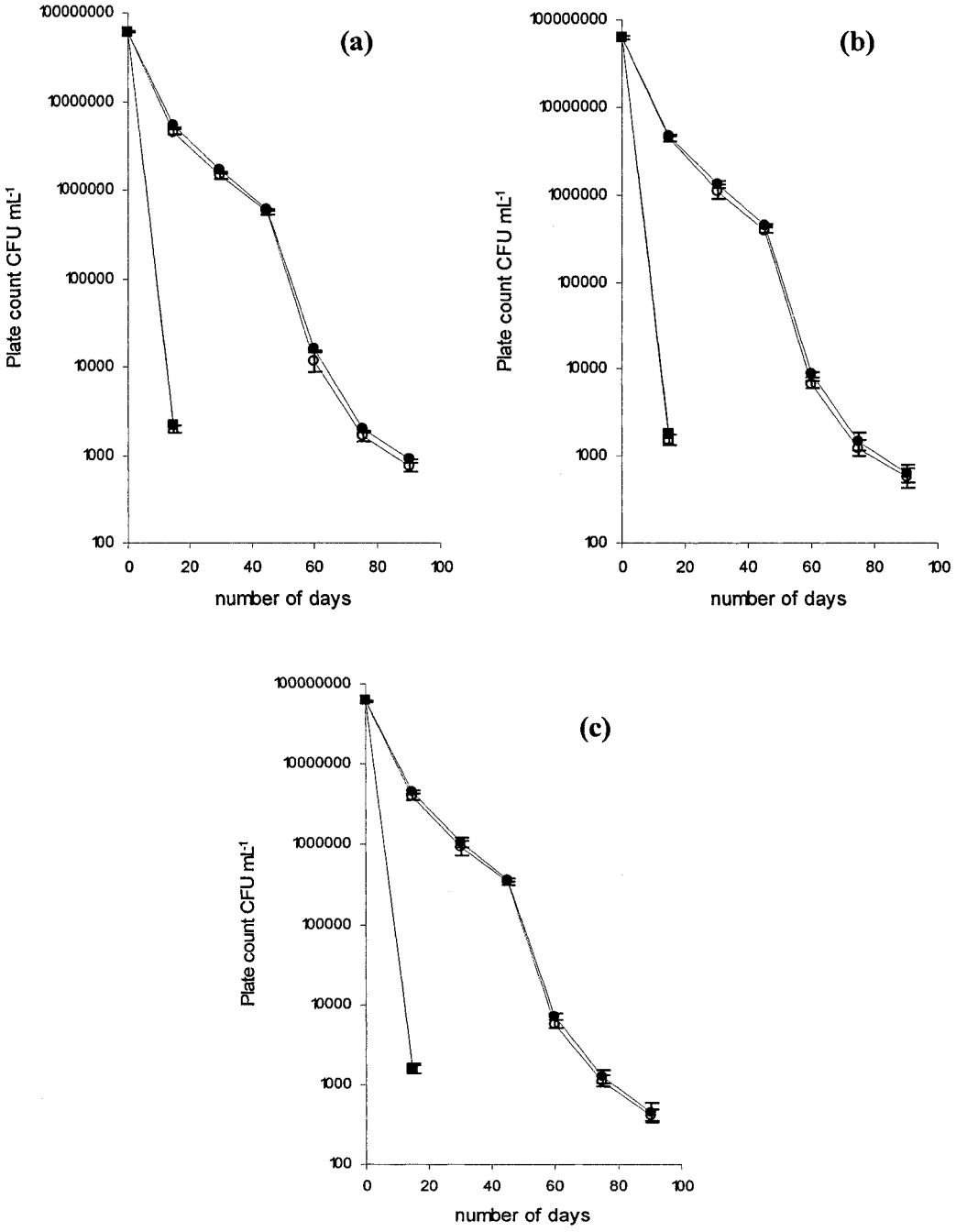


Fig. 4.21 Effects of long-term incubation in sterile distilled water on the enumeration of *E. faecalis* NCTC775 at 25°C and 37°C (a) nutrient agar, (b) KF streptococcus agar and (c) Slanetz and Bartley agar incubated either, under aerobic conditions (open symbols), or ROS-neutralised conditions (closed symbols) with incubation temperatures of 25°C (circles) and 37°C (squares). The initial inoculum is represented as start point on Y-axis. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

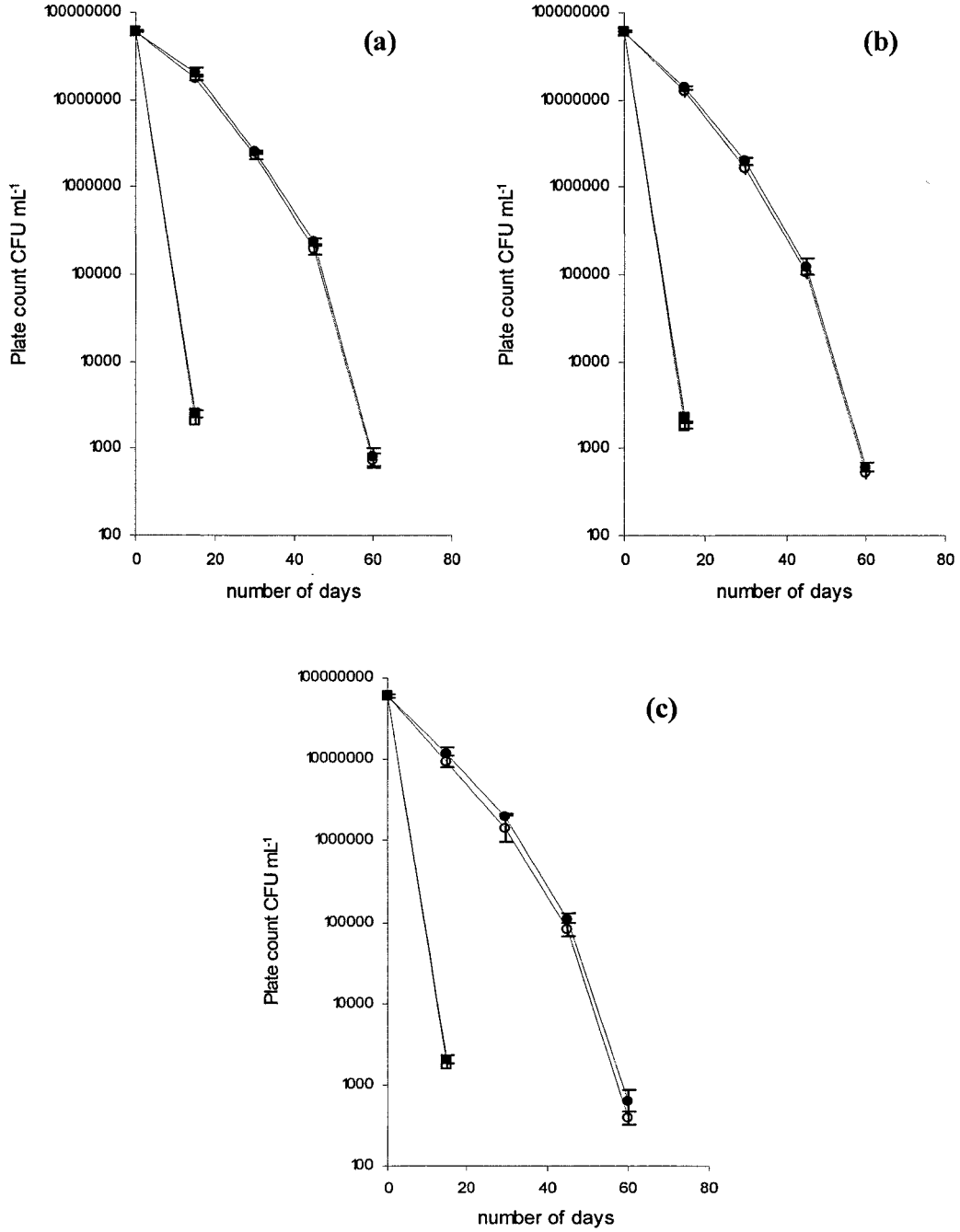


Fig. 4.22 Effects of long-term incubation in sterile distilled water on the enumeration of *E. faecalis* ATCC35550 at 25°C and 37°C (a) nutrient agar, (b) KF streptococcus agar and (c) Slanetz and Bartley agar incubated either, under aerobic conditions (open symbols), or ROS-neutralised conditions (closed symbols) with incubation temperatures of 25°C (circles) and 37°C (squares). The initial inoculum is represented as start point on Y-axis. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

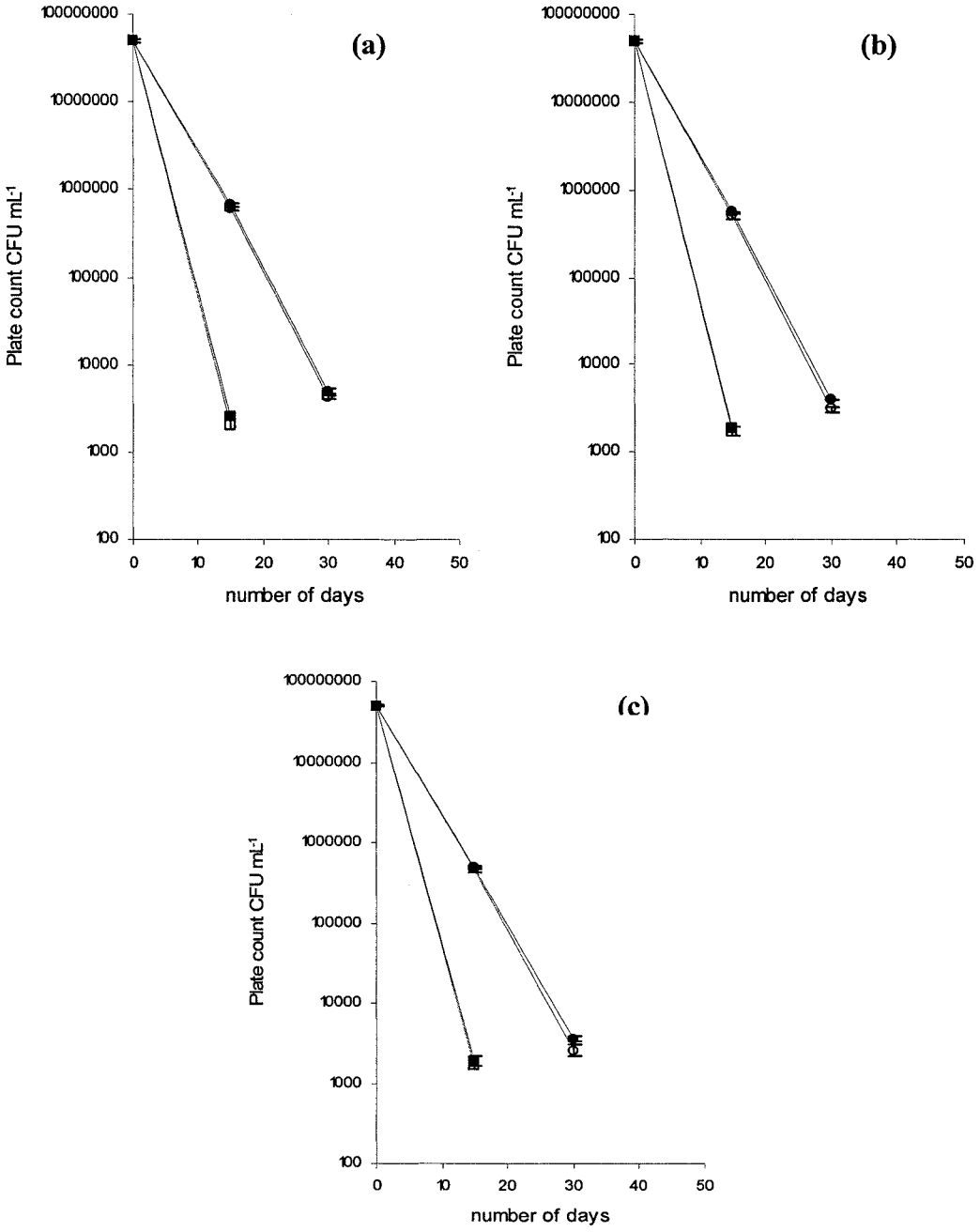


Fig. 4.23 Effects of long-term incubation in sterile distilled water on the enumeration of *E. faecalis* PTO1 at 25°C and 37°C (a) nutrient agar, (b) KF streptococcus agar and (c) Slanetz and Bartley agar incubated either, under aerobic conditions (open symbols), or ROS-neutralised conditions (closed symbols) with incubation temperatures of 25°C (circles) and 37°C (squares). The initial inoculum is represented as start point on Y-axis. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

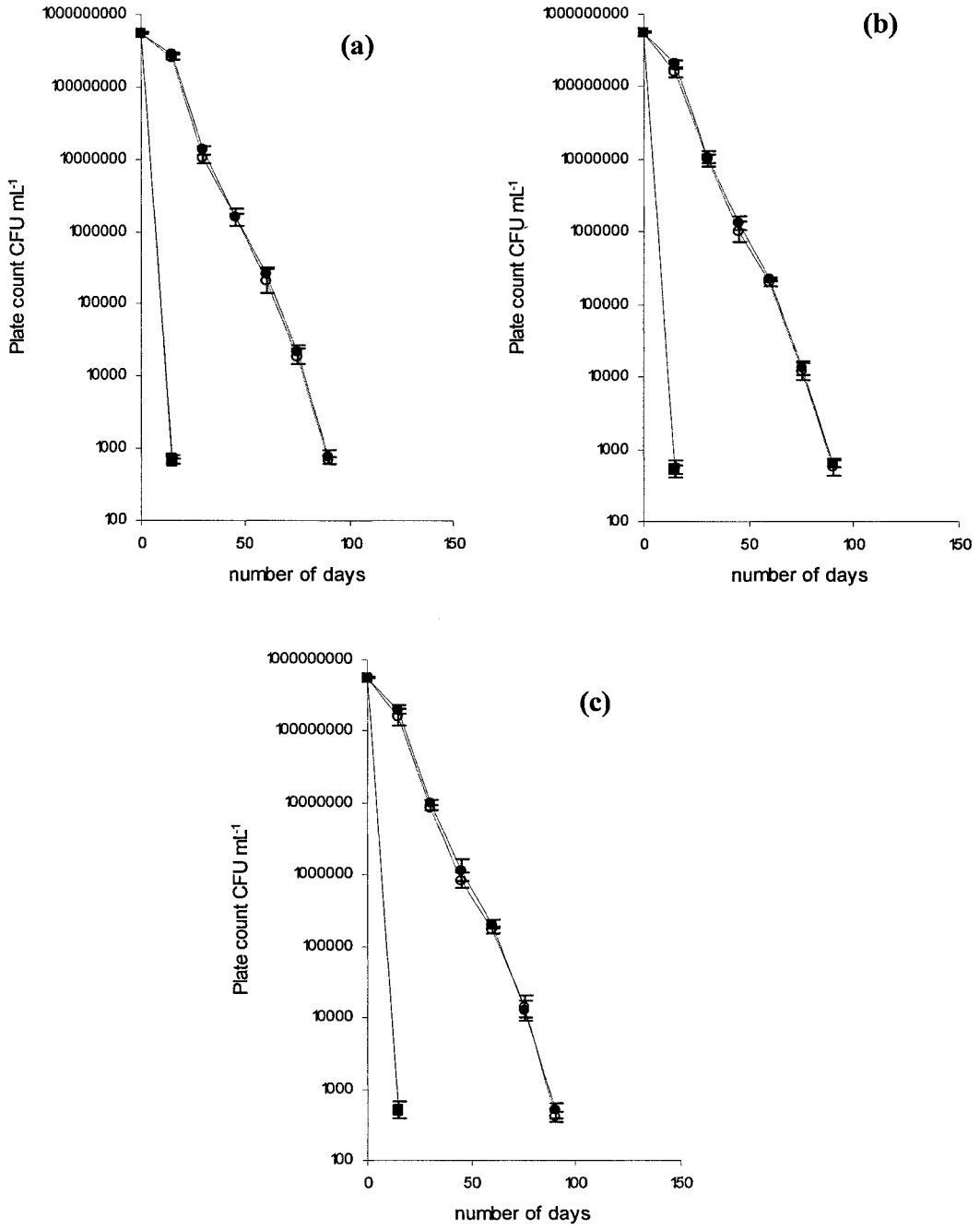


Fig. 4.24 Effects of long-term incubation in sterile distilled water on the enumeration of *E. faecalis* PTO2 at 25°C and 37°C (a) nutrient agar, (b) KF streptococcus agar and (c) Slanetz and Bartley agar incubated either, under aerobic conditions (open symbols), or ROS-neutralised conditions (closed symbols) with incubation temperatures of 25°C (circles) and 37°C (squares). The initial inoculum is represented as start point on Y-axis. Error bars represent 95% confidence limits (n=3). Note that the vertical axis is log-transformed.

4.4 Discussion

Exposure of faecal indicator bacteria present in contaminated drinking water systems to various physico-chemical factors, for example during disinfection of water, is known to produce sub-lethal injury (McFeters *et al.*, 1986; Clesceri *et al.*, 1998; Bjergbaek and Roslev, 2005), which can affect their ability to grow on the selective media predominantly used for assessment of water quality (Brenner *et al.*, 1996; Chilvers *et al.*, 2001). Loss of culturability of bacteria during their survival in aquatic systems (Gourmelon *et al.*, 1994), in potable water (McFeters and Singh, 1991), and in sewage treatment systems (Curtis *et al.*, 1992) has been studied. The failure to detect and thus enumerate sub-lethally injured/debilitated bacteria such as faecal indicators could lead to underestimation of their true levels in drinking water (Calabrese and Bissonette, 1990a).

The antimicrobial properties of sunlight have been known well over a century but its practical use for disinfecting drinking water comes from only 25 years ago (Acra *et al.*, 1980; Patwardhan, 1990). Since then sunlight has been studied for the inactivation of several bacteria including faecal indicator bacteria in fresh waters (Kussovski *et al.*, 2001), waste waters (Koivunen and Heinonen-Tanski, 2005), sewage (Fujioka *et al.*, 1981) and drinking water (Reed *et al.*, 2000). Sunlight has been known to eliminate micro-organisms such as viruses, enteric bacteria such as *E. coli* and *E. faecalis* and *Salmonella enteritidis* (Koivunen *et al.*, 2003; Rajala *et al.*, 2003; Lonnen *et al.*, 2005). Most solar disinfection studies have been conducted with either *E. coli* or *E. faecalis,* by enumeration onto growth medium incubated under standard aerobic conditions but recent research has suggested that such conditions are effective in growing healthy cells

only (Reed, 2004). The effects of oxygen in inhibiting sunlight-irradiated cells has been studied recently, where reactive oxygen species (ROS) have been neutralised by adding quenchers of peroxides such as pyruvate and catalase (Kehoe, 2001, Reed, 2004) and/or by incubation of agar plates under anaerobic conditions (Khaengraeng and Reed, 2005). In the present study a similar type of oxygen toxicity was seen when *E. coli* and *E. faecalis* strains were illuminated under UV-A or natural sunlight (Table 4.1, Fig. 4.1, 4.2a-c, Fig. 4.3 and 4.4a-c). Maximum counts for UV-A exposed *E. coli* NCTC8912 were noted under ROS-neutralised conditions, followed by peroxide-neutralised conditions and then under anaerobic conditions, with lowest count observed under aerobic conditions (Table 4.1). The results indicate that injured ROS-sensitive cells may be unable to grow under aerobic conditions, with peroxide-neutralised conditions increasing the count but unable to detoxify all of the reactive oxygen species as further enhancement in counts were obtained under ROS-neutralised conditions. In the case of selective media (Fig. 4.1) under aerobic conditions, MacConkey agar proved to be least inhibitory followed by mFC-R and mEndo with the lowest count obtained using mLSA. Enhancement in counts was observed on all selective media except mEndo under ROS-neutralised conditions. The results suggest that the inhibitory ingredients in mEndo medium such as pararosanilin or sodium deoxycholate limit the growth of injured bacteria and thereby act as sources of secondary stress (Chilvers, 2001; Khaengraeng, 2004). In contrast to the results for *E. coli*, *E. faecalis* NCTC775 (Fig. 4.3) showed a smaller effect of ROS neutralisation on all sets of growth media (non-selective or selective agar). The non-selective medium gave higher counts than corresponding selective media under aerobic and ROS-neutralised conditions. The inhibitory effect of

different growth media on enumerating sub-lethally injured bacteria has been studied previously; for example Fujioka and Narikawa (1982) noted reduced colony formation for faecal coliforms (on mFC agar) and faecal streptococci (on KF streptococcus agar) after exposure to sunlight as compared to non-selective nutrient agar. Furthermore it has been noticed that sunlight-illuminated cells of *E. coli* may give false-negative results on selective mFC agar when compared with non-selective nutrient agar (Shah *et al.*, 1996; Khaengraeng, 2004). All of these studies support the results obtained in the present study for different strains of *E. coli* and *E. faecalis* (Figs. 4.1-4.4), where reduced counts were seen in selective media for UV-A-treated or sunlight-irradiated cells. It has been explained in Chapter 3 that oxygen toxicity and the production of ROS may play a key role in decreasing the cell count under conventional enumeration conditions.

High temperatures have been known to cause sub-lethal injury by damaging microbial cell components at the molecular level (Andrews and Ray, 1989; Kang and Siragusa, 1999) with increase of cell surface hydrophobicity, inactivation of cell enzymes/RNA/DNA and subsequent loss of cell viability (Clavero and Beuchat, 1996; Czechowicz *et al.*, 1996). It has been reported that heat-injured bacteria such as faecal coliforms and enterococci become more susceptible to secondary stress due to selective agents present in growth media (McDonald *et al.,* 1983; Rince *et al.*, 2003) which supports the results obtained in present study (Fig 4.5, 4.6 and Fig. 4.7 and 4.8). Heat-treated cells of *E. coli* showed a reduction in count when grown on conventional growth medium in the presence of oxygen, i.e. under standard aerobic conditions, and the counts were increased when these injured cells were given anaerobic conditions. These results

are in agreement with those obtained by Bromberg *et al.* (1998) and George *et al.* (1998). These workers carried out experiments to find out the effect of oxygen concentration and redox potential on the recovery of sub-lethally heat-damaged cells of *E. coli* O157, *Salmonella enteritidis* and *Listeria monocytogenes,* supporting the concept that growth of injured bacteria under anaerobic conditions can give higher counts than under aerobic conditions. On the basis of these experiments they concluded that there was an approximately eight-fold greater heat resistance when these bacteria were grown, heated and recovered anaerobically rather than aerobically. Ugborogho and Ingham (1994) recovered more heat-injured *Staphylococcus aureus* when the enrichment medium was incubated under strict anaerobic conditions. A similar enhancement in aerobic count was noticed for *E. coli* NCTC8912 as shown in Table 4.2 with incubation of cells under anaerobic conditions. Other studies on *Salmonella sp.* have suggested that reactive oxygen species (ROS) produced following sub-lethal heat treatment inhibit growth on selective media which can be counteracted by addition of quenchers of ROS (Stephens *et al.,* 2000) including catalase and pyruvate, which detoxify H_2O_2 (Czechowicz *et al.*, 1996). These quenchers of ROS either degrade or block the formation of reactive oxygen species such as H_2O_2 and superoxide. (McDonald *et al.,* 1983) Thus an increase in count obtained for *E. coli* NCTC8912 in the presence of pyruvate (Fig. 4.5) from its aerobic counterpart is likely to be due to the quenching of peroxides. Oxyrase which has both enzymes to degrade ROS and the ability to turn a broth-based medium anaerobic has been used for the enumeration of anaerobic organisms such as *Clostridium perfringens* (Stephens *et al.,* 2000). An enhancement in count was obtained for heat-injured *E. faecalis* NCTC775 under ROS-neutralised

conditions, namely supplementation of the growth medium with pyruvate and incubation under anaerobic conditions (Fig. 4.7) when compared with the corresponding aerobic count as was also noted for *E. faecalis* stored in water kept in a brass vessel (Chapter 3, Fig. 3.13).

Chlorination of water supplies and polluted water is designed primarily to destroy or inactivate pathogenic micro-organisms (Calabrese and Bissonnette, 1990b; Clesceri *et al.*, 1998). It is well recognized that short-term exposure to low levels of free chlorine (typically 0.2-2.0 $\mu g\ ml^{-1}$) is lethal only for a fraction of the exposed bacteria but induces metabolic injury to a large proportion of the cells, often limiting their growth on selective media (Lechavallier and McFeters, 1985; Tryland *et al.,* 1998; Rizzo *et al.,* 2004). For example, experiments on chlorine-injured *E. coli* showed decreased oxidation of glucose and damage to enzymes involved in carbohydrate metabolism (Jacangelo *et al.,* 1987; Tosa *et al.,* 1997) whereas others have reported damage to the bacterial cell membrane and associated transport processes, leading to loss of culturability (Lisle *et al.,* 1999). Such damage is also consistent with the overall findings of the present study. Thus, after chlorine disinfection the bacteria can be envisaged as being in one of three states: firstly, healthy cells which are not substantially injured and which can be enumerated on any growth medium or conditions; secondly, cells injured to varying degrees, which can be cultured on agar-based media only under the most favorable conditions (Table 4.3, Figs. 4.9, 4.10, 4.11 and 4.12); and thirdly, cells that are inactivated (Tosa *et al.,* 1997). These states have been described in more detail in Chapter 3 (Fig. 3.16).

The earlier work of Calabrese and Bissonnette (1990b) demonstrated that chlorine-stressed coliforms could give enhanced counts on pyruvate-supplemented media. Such enhancement was also observed in the present study and can be explained by the well-established peroxide-scavenging effect of pyruvate (Czechowicz *et al.* 1996; Stephens *et al.* 2000). Rompre *et al.* (2002) highlight suggestions by several researchers that chlorination may have a specific adverse effect on cellular catalase activity. Dukan and Tonati (1996) have indicated that catalase neutralises the effect of hypochlorite (HOCl) or its derivatives and have mentioned that there could be a synergistic effect between damage produced by HOCl or its derivatives and an increase in the concentration of H_2O_2 in catalase-deficient mutants of *E. coli.* While an increase in counts on supplementing the medium with pyruvate was observed for several different media and strains of *E. coli* and during the present study (Table 4.3, Figs. 4.9, 4.10), a further enhancement was obtained on incubation of pyruvate-supplemented medium under anaerobic conditions, demonstrating that formation of peroxides alone is not the sole reason for the reduction in counts observed under standard aerobic conditions, and that ROS-neutralised conditions are required for more effective enumeration of chlorine-damaged *E. coli.* The results are also consistent with the hypothesis that ROS-induced self-destruction is responsible for a substantial lowering of the counts of chlorine-injured *E. coli* in a range of media, since this would be eliminated under anaerobic conditions. However, the fact that the ROS-neutralised counts of the various selective media still show differences indicates that the selective agents used in each medium still exhibit a differential toxicity towards injured cells, with media such as MacConkey agar generally

showing the least evidence of toxicity (highest counts) while other media, e.g. lauryl sulphate agar (mLSA) show a greater inhibition.

For *E. faecalis* a reduced effect of ROS-neutralisation was seen on non-selective media compared to *E. coli*, which may be due to the fact that *E. faecalis* tolerates and grows in presence of oxygen as it is an aerotolerant anaerobe (Flahaut *et al.*, 1998) and therefore lacks respiration-induced self-destruction as a source of sub-lethal injury. Nevertheless, the results obtained for different strains of chlorine injured *E. faecalis* (Fig. 4.11 and 4.12a-c) consistently show a reduced aerobic count on all sets of growth media compared to ROS-neutralised conditions. The results indicate that selective media such as MacConkey agar number 2, containing bile salts number 2, are most inhibitory to the growth of chlorine-injured *E. faecalis* cells under aerobic conditions, followed by KF streptococcus agar (Flahaut *et al.*, 1996; Rince *et al.,* 2003), with an enhancement in counts on all media under ROS-neutralised conditions. Thus all of the inhibitory effects of selective media cannot be countered simply by neutralisation of ROS.

Low pH is one of the most important environmental factors affecting growth and has been studied mostly because of its importance in fundamental research and its practical importance in biotechnology, as well as in natural environments (Rosso *et al.*, 1995). Acid resistance and tolerance of bacteria such as *E. coli* O157 (Benjamin and Datta, 1995; Diez-Gonzalez and Russell, 1999) and *Enterococcus faecalis* (Rince *et al.*, 2000) at low pH conditions similar to those of the human stomach have been studied to understand acid tolerance responses (e.g. Brown *et al.*, 1997; Koutsoumanis and Sofos,

2004). Acid mine water is characterized by low pH and elevated concentrations of metal ions. This water has been noted to induce sub-lethal injury to *E. coli* and other coliform bacteria (Wortman and Bissonnette, 1985). Calabrese and Bissonnette (1990b) suggested that exogenous supplementation of pyruvate or catalase in the growth medium could lead to improved detection of injured cells in acid mine water. In the present study using *E. coli* (Table 4.4, Fig 4.13 and 4.14 a-c) and *E. faecalis* (Fig. 4.15 and 4.16 a-c) no major difference between counts on, non-selective and selective media under aerobic or ROS-neutralised conditions was observed which might be explained by the fact that pH as a single factor was studied without combination with other factors such as metal ions. One interpretation of these results is that exposure to low pH decreased the bacterial count but the damage was predominantly irreversible and not sub-lethal, since almost equivalent counts were obtained irrespective of the growth conditions or media used.

Most microbiology laboratories engaged in research, teaching or in industry need to preserve or store bacterial cultures for short periods, for example by immersing in mineral oil (Heckly, 1978; Park *et al.,* 2001) and for long periods by freeze-drying or ultra-freezing (Gherna, 1994). Furthermore it has been reported that phytopathogenic bacteria can be maintained in pure water for several years (van Elsas *et al.*, 2001). Liao and Shollenberger (2003) studied the survival and long-term preservation of bacteria in water and in phosphate buffered saline (PBS). They concluded that different Gram-negative bacteria could be preserved in water for at least several months to several years whereas Gram-positive bacteria survive better in PBS than in water. Similarly Hartke *et al.* (1998) and Signoretto *et al.* (2000) studied the changes in cell morphology of

Enterococcus faecalis in oligotrophic environments and concluded that this organism could survive for prolonged periods under conditions of complete starvation during incubation in tap water. Describing the results obtained for different strains of Gram-negative *E. coli* stored in distilled water at temperatures 25°C and 37°C in Figs. 4.17-4.20a-e it is clear that the rate of inactivation depended upon the storage temperature as the bacterial strains gave higher counts when stored for long periods at a temperature of 25°C than at 37°C. Similar results were also obtained for the Gram-positive bacterium *Enterococcus faecalis,* as shown in Figs. 4.21-4.24. Roslev *et al.* (2004) studied the effect of oxygen on the survival of faecal indicators in drinking water, and they concluded that oxygen is a key regulator of the survival of *E. coli* in non-disinfected water and stated that these organisms persisted longer in drinking water under anaerobic conditions. Subsequently, the same group examined the difference in counts obtained using traditional culture-based methods and culture-independent methods (DVC, FISH) with a resuscitation step to study the survival and incubation of *E. coli* strains in drinking water (Bjergbaek and Roslev, 2005). In the present study similar count was obtained on nutrient agar and various selective media used for enumeration of *E. coli* (Figs. 4.17- 4.20 a-e) and *Enterococcus faecalis* (Figs. 4.21- 4.24 a-c) at storage temperatures of 25°C and 37°C. In contrast, Bogosian *et al.*, (2000) studied the viability of storing *Vibrio vulnificus* in seawater at low temperatures. Midway in their study, they observed a 1000-fold higher count on growth medium supplemented with catalase or pyruvate compared with corresponding unsupplemented growth medium. This contrasts with the results of the present study where *E. coli* and *E. faecalis* stored at high temperatures (25°C and 37°C) in the distilled water (pH=5) showed no increase on

growth medium incubated under ROS-neutralised conditions, indicating irreversible inactivation rather than sub-lethal injury.

Based on the current results, it would seem that stressors such as sunlight, temperature and chlorine may induce sub-lethal injury in bacterial cells of *E. coli* and *E. faecalis*. The sub-lethal injury may inactivate the bacterial cells under conventional aerobic enumeration conditions as a result of oxygen-sensitivity by inactivating their enzyme mechanism (Ugborogho and Ingham, 1994; Reed, 2004). A further possible source of ROS may be produced within the growth medium is light dependent, due to the presence of photosensitize components of the growth medium, such as riboflavin and tryptophan, which may enhance the generation of ROS (Grzelak *et al.*, 2001; Spiegeleer *et al.*, 2004) in the presence of light. Thus bacterial cells could be envisaged to be in any of the three states as a result of sunlight, chlorine or temperature injury which have been discussed in detail in Chapter 3 (Fig. 3.16). However, the injury produced due to the other stressors namely pH and starvation are not strongly oxygen sensitive as no differences between the counts were observed on various growth media or enumeration conditions. The bacterial cells in starvation experiments were stored in distilled water which has a pH of 5. They could be maintained in such weakly acidic distilled water for only a period of a few weeks. The storage in weakly acidic distilled water showing slow decrease in counts could be envisaged as a very mild form of acid stress and thereby linked to experiments in distilled water maintained at low pH value of 2 where there is a rapid decrease in count. The mechanism of injury induced in bacterial cells at low pH or weak acidic distilled water (starvation) after short periods of storage might be by irreversibly

denaturing proteins or by inactivating acid resistant mechanisms which could not be renatured or activated even after providing favorable growth conditions or growth media. The acid resistance varies in different strains of *E. coli*, for example *E. coli* O157 a pathogenic strain, is highly resistant to low pH (Jordan *et al.*, 1999a; Large *et al.*, 2005). Most studies investigating inactivation of *E. coli* at low pH have examined the survival rate using conventional tryptic soya agar under aerobic conditions (e.g. Takumi *et al.*, 2000). Other studies have focused on inactivation of *E. coli* by the combined effects of number of factors, of which pH is only one aspect (Jordan *et al.*, 1999b). Thus little information is available regarding the affect of low pH as a sole factor for the inactivation of *E. coli* and its enumeration under different sets of growth conditions or growth media. Summarizing the results obtained in starvation and low pH, the bacteria in these conditions could be envisaged to be present in two states, firstly healthy which could be grown on any growth medium or enumeration conditions and secondly inactivated which cannot be grown on any growth medium or enumeration conditions, with little evidence of sub-lethal injury in these studies.

Chapter 5

Resuscitation and pre-enrichment of faecal indicator bacteria enumerated from environmental water samples

5.1 Introduction

Resuscitation has been defined as a growth-enrichment step resulting in an injured bacterial cell attaining the capability to be culturable on those media which are normally employed for their growth, or the conversion of nonculturable bacterial cells impaired as a result of deformities induced in metabolic and physiological processes into culturable cells (Oliver, 1993; Stephens *et al.*, 2000; Oliver, 2005). Faecal indicator bacterial cells may be subjected to various sub-lethal stresses (Rochelle *et al.*, 1996; Kang and Fung, 1999; Kang, 2002; Reed, 2004) prevalent in the environment, such as in drinking water (Brenner *et al.*, 1996; Khaengraeng, 2004) or associated with partial or inadequate disinfection (Domek *et al.,* 1984; Singh *et al.*, 1990; McFeters *et al.*, 1995). These stresses may leave the bacteria in a debilitated state (Campher *et al.*, 1979; Singh *et al.*, 1986; Tosa *et al.,* 1997). These debilitated or sub-lethally injured bacteria may thus lose the ability to grow on media in routine used that are otherwise satisfactory for cultivation of healthy cells (Rizzo *et al.,* 2004; Spiegeleer *et al.,* 2004). Such sub-lethal injury could be ascribed to selective agents present in the growth media. Many selective agents such as pararosaniline and sodium sulphite present in m-Endo (Sciff *et al.*, 1970; Singh *et al.*, 1990; Chilvers, 2001), bile salts in MacConkey agar (McFeters *et al.*, 1986; Sartory, 1995), sodium deoxycholate in xylose lysine deoxycholate agar (Kang, 2002), rosolic acid in membrane faecal coliform agar (mFC) a standard US faecal indicator medium (Clesceri *et al.*, 1998), sodium lauryl sulphate in membrane lauryl sulphate agar (mLSA) a standard UK faecal indicator medium (Babitch and Babitch, 1997), and sodium azide in Slanetz and Bartley agar (Audicana *et al.*, 1995) and KF streptococcus agar (Fujioka and Narikawa, 1982) act as a source of secondary stress for bacterial growth. Thus different selective media can recover and enumerate stressed bacteria to varying efficiencies (Tosa *et al.*, 1997) and differences in counts

between non-selective and selective media can be used as a measure of the degree of injury (Kang and Siragusa, 1999; see also Chapters 3 and 4).

Different approaches have been used to culture injured bacteria, including faecal indicators. Resuscitation of stressed or injured bacteria has been enhanced by inoculating and culturing samples initially in an enriched, non-inhibitory medium at moderate temperatures, for example a 2 hour incubation at 35°C followed by incubation at 44.5°C for 18-22 hour for total coliforms using mFC agar (Clesceri *et al.*, 1998) or 2 hour incubation at 37°C followed by incubation for 46 hour for enterococci using Slanetz and Bartley agar (Reuters, 1985; Domig *et al.*, 2003). Alternatively, incorporation of a pre-enrichment step using a non-selective nutrient-rich media such as nutrient agar or phenol red lactose broth for faecal coliforms or tryptic soya agar for enterococci for 2-4 hour at incubation temperatures of 35°C followed by 20-22 hour incubation at 44.5°C for faecal coliforms or 46 hour for enterococci using selective media (Lin, 1976, Steen and Eie, 1992; Domig *et al.*, 2003). Enhanced resuscitation of heat-injured bacteria has also been noted by researchers using a two-layer agar method consisting of non-selective agar such as lactose broth overlayed onto selective mFC agar (Rose *et al.*, 1975; Han *et al.*, 2002). Similarly, Kang and Siragusa (1999) have tested the agar underlay method using two-chambered Petri dish having a non-selective agar layer poured into the top chamber of the Petri dish for 2 hour as an initial step for the revival of injured bacteria followed by a selective agar layer in the bottom chamber, for selectivity.

Furthermore, a modified selective medium named as plate count-monsensin-KCl (PMK) agar with the ionophore such as monensin, the surfactant Tergitol and with the diagnostic agent 4-methylumbelliferyl-β-glucuronide (MU-GLUC) have been

used for the resuscitation of injured coliform bacteria from sewage and surface waters (Freier and Hartman, 1987).

Currently used approved culture-based methods rely on traditional most probable number (MPN) and membrane filtration (MF) technique that have been criticized for many shortcomings (Hartman *et al.,* 1992; Rompre *et al.*, 2002); for example the use of lauryl tryptose broth or lactose broth can lead to interference from high numbers of non-coliform bacteria in the case of MPN (Evans *et al.*, 1981; Seidler *et al.*, 1981) and the formation of typical colonies with a sheen on mEndo agar can also be produced by non-coliforms in the case of MF, with high number of background heterotrophic bacteria decreasing the overall resuscitation of coliforms (Clark, 1980; Dufour *et al.*, 1981; Burlingame *et al.*, 1984; Rompre *et al.*, 2002). Improvements have been introduced in the traditional methods to reduce incubation time and to increase specificity, sensitivity and selective detection, for example the use of defined substrate technology in traditional culture-based methods. Edberg and Edberg (1988) refined the MPN method into a single step presence-absence test by incorporating a substrate *o*-nitrophenyl-β-galactopyranoside (ONPG), specific for the enzyme β-galactosidase present in all coliforms, and the substrate MU-GLUC for the specific detection of *Escherichia coli* having the enzyme β-glucuronidase. Similarly, Brenner *et al.* (1993) introduced a new MF medium called MI agar containing a chromogen, indoxyl-β-glucuronide and a fluorogen 4-methylumbelliferyl-β-galactopyranoside (MU-GAL) for the rapid detection and enumeration of both total coliforms (TC) and *Escherichia coli* in water samples. On the same principles new agar and broth-based media introduced in MPN and MF format have been proposed for enterococci for fast enumeration from water, for example the addition of indoxyl-β-glucoside to mEI agar (Messer and Dufour, 1998).

Many researchers have studied the comparative effectiveness of MPN and MF techniques for improving the enumeration of *Escherichia coli* (McFeters *et al.*, 1982; McFeters *et al.*, 1995) and enterococci (Volterra *et al.*, 1985; Massa *et al.*, 2001) from water samples; for example Colilert (IDEXX, Westbrook, Maine) and Enterolert (IDEXX, Westbrook, Maine) which are in MPN format have been compared with the same growth media used in the MF format (Eckner, 1998). Mossel *et al.* (1980) studied the differences obtained between a liquid repair medium as a resuscitation step with agar-based MacConkey medium supplemented with catalase for the resuscitation of sub-lethally injured bacteria belonging to the family Enterobacteriaceae. In the present Chapter, experiments were designed to establish the relative importance of a resuscitative medium or a pre-enrichment step compared with the use of a ROS-neutralised medium for the enumeration of sub-lethally injured total coliforms and enterococci from environmental samples.

The specific objectives of the present study were:

1. To compare a resuscitative medium (m-T7 agar) for total coliforms (TC) and/a pre-enrichment step prior to use of a selective medium for enterococci with the use of ROS-neutralised selective medium for the detection and enumeration of these organisms from environmental samples using the membrane filtration technique.
2. To investigate differences in the count obtained for faecal indicator bacteria enumerated from the environmental samples using a broth-based most probable number method and an agar-based membrane filtration method using standard aerobic unsupplemented medium and ROS-neutralised conditions.

5.2 Materials and Methods

Water samples were collected from various environmental sources in Panjab, India such as handpumps, taps, water coolers and rivers, aseptically in sterile bottles. The samples were processed using most probable number (MPN) and membrane filtration (MF) techniques for the selective isolation and enumeration of the faecal coliforms and enterococci as described in Chapter 2.

5.2.1 Comparison of resuscitation media (total coliforms) and pre-enrichment (enterococci) for the enumeration of faecal indicator bacteria in water samples

Total coliforms Selective enumeration of faecal coliforms was carried out using a commercial resuscitative medium m-T7 agar (LeChevallier *et al.*, 1983; Clesceri *et al.*, 1998), and several standard selective media namely, MacConkey agar, m-lauryl sulphate agar, m-faecal coliform agar without rosolic acid, and m-Endo agar (Labchem Himedia, Mumbai, India). The selective agents present in MacConkey agar, m-Lauryl sulphate agar, m-Faecal Coliform agar without rosolic acid, and m-Endo agar have been described in detail in Chapter 2. The media were enumerated using aerobic conditions (unsupplemented media) and ROS-neutralised conditions (growth media supplemented with 0.05% w/v sodium pyruvate and incubated in an anaerobic jar). The typical composition of m-T7 agar in g L^{-1} is 5 g of proteose peptone number 3, 0.3 g of yeast extract, 20 g of lactose, 0.4 mL of Tergitol 7, 5 g of polyoxyethylene ether W1, 0.1 g of bromthymol blue, 0.1 g of bromocresol purple and 15 g of bacteriological agar. An amount of 1.0 µg of penicillin G per mL was aseptically added to the medium after autoclaving and after cooling the media to 45°C for additional selectivity. Typical yellow colonies formed after incubation of plates at 35°C for 24 h (LeChevallier *et al.*, 1983; LeChevallier *et al.*, 1984; Clesceri *et al.*, 1998) were counted and expressed as CFU 100 mL^{-1}. Less contaminated water

samples between 1-100 mL were processed by MF technique whereas the spread plate technique was applied to those samples that were highly contaminated and needed 0.02-0.5 mL of sample to be processed. Comparisons were made between the resuscitation medium m-T7 agar and various other selective media under aerobic and ROS-neutralised conditions to note the performance and difference in total coliform count for each medium after incubation of triplicate plates as described in Chapter 2.

Statistical analysis was carried out using the Wilcoxon (matched pairs) signed rank test (Kirkwood *et al.*, 2003) because the two methods which were to be compared do not give directly equivalent numerical values, for example in the case of plate counts or the MF method, CFU 100 mL^{-1} was obtained which are an exact count whereas in the case of MPN method, MPN $100mL^{-1}$ was calculated which gives only a probability or estimate of the value on a discontinuous scale. . The Wilcoxon sign rank test is the non-parametric equivalent of the paired *t* test. In this test the sign and relative magnitude of the data but not the actual values are used.

For example, consider the results (worked example) shown in Table 5.1, where the MPN values calculated using two media for 10 samples are compared by calculating differences between the two, using the four basic steps of the Wilcoxon signed rank test, as follows:

1. All differences that were zero were excluded. The remaining differences were placed in ascending order after ignoring their signs and ranking them as 1, 2, 3, etc. The differences that were equal were averaged by ranks as in the case of samples numbered 4 and 7. The ranks are shown in Table 5.1.
2. Ranks of positive and negative differences were counted and their sum denoted by T_+ and T_- respectively.

$T_+ = 6$ (only rank value)

$T_- = 8 + 9 + 3.5 + 2 + 1 + 3.5 + 7 + 5 + 10 = 49$ (9 values).

3. If there were no substantial difference between the two media then the absolute values of T_+ and T_- would be similar. Conversely, where there is difference between the two then one sum is likely to be smaller and the other to be larger than expected. T denotes the smaller sum.

   T = smaller of T_+ and T_-

   So T = 6 in this example.

4. The Wilcoxon signed rank test then assesses whether T, the smaller of the two values is smaller than the critical value, based on tabulated values at a particular probability (Kirkwood *et al.*, 2003) and the number of ranked (non-zero) differences (N).

Thus in this example according to Table 5.1, N =10 and T =6, which is lower than the corresponding critical value of 8 (P=0.05). In this example, it can be concluded that the two media give significantly different MPN values (in this instance, the values for the medium 2 are consistently higher than for medium 1).

Table 5.1 Worked example of Wilcoxon signed rank test

Sample Number	MPN medium 1	MPN medium 2	Difference	Rank (ignoring sign)
1	21	120	-99	8
2	75	230	-155	9
3	247	230	17	6
4	4.7	12	-7.3	3.5*
5	2.1	4.7	-2.6	2
6	1.2	2.1	-0.9	1
7	4.7	12	-7.3	3.5*
8	47	120	-73	7
9	12	21	-9	5
10	230	550	-320	10

* 4^{th} sample and 7^{th} sample tied, and so averaged.

Enterococci These were selectively enumerated from water samples by the MF technique using commercial media namely KF streptococcus agar and Slanetz and Bartley agar. The growth media were incubated under aerobic conditions (unsupplemented media) conditions and ROS-neutralised conditions. A pre-enrichment step of 3 hour with transfer of the membrane-filter onto Millipore absorbent pads (Millipore Corporation, Consett, UK) pre-soaked with bile broth medium and incubated at 35°C (Lin, 1974; Clesceri *et al.*, 1998). The bile broth medium consisted of broth prepared by adding 40 mL of sterile 10% oxgall or oxbile to 60 mL of sterile brain heart infusion broth (Labchem Himedia, Mumbai, India). After the pre-enrichment step the membrane filters were carefully placed on the selective media described above with further incubation at 35°C for 48 h. Comparisons for the counts obtained for enterococci were made between the membranes directly placed on selective media and those involving the pre-enrichment step, and then placed on selective media under aerobic and ROS-neutralised conditions.

5.2.2 Comparisons of standard most probable number (MPN) technique and membrane filtration (MF) technique for the enumeration of faecal indicator bacteria in water samples

Escherichia coli MacConkey broth medium (Labchem Himedia, Mumbai, India) was used for comparison between MPN assay and MF assay. In the MF method, 15 g L^{-1} of bacteriological agar was added to MacConkey broth medium. MPN broth tubes and MF-based unsupplemented agar plates were incubated under standard aerobic conditions, while MF agar plates supplemented with 0.05% w/v sodium pyruvate were incubated under anaerobic conditions in an anaerobic jar (ROS-neutralised conditions). All of the growth media used was incubated at 37°C for 48 h. After 48 h, confirmation of positive acid-gas forming MPN tubes and typical yellow-orange colonies on MF plates indicating presumptive total coliforms were streaked on HiChrome *E. coli* agar (Labchem Himedia, Mumbai, India), with β-galactosidase and β-glucuronidase-positive faecal coliforms (*E. coli*) giving purple coloured colonies and β-galactosidase-positive coliforms giving red coloured colonies.

***Enterococcus* spp.** In the MPN assay, azide dextrose broth (Labchem Himedia, Mumbai, India), initially recommended in 1948 and used since then in US standard methods (Clesceri *et al.*, 1998) for the MPN procedure in a quantity of 34.7 g L^{-1} was selected. It consists of sodium azide as the main selective agent for the inhibition of Gram-negative aerobic and facultative anaerobic bacteria with additional sodium chloride. Beef extract, tryptone and glucose in the broth serve as nutritional sources. In MF assay, Slanetz and Bartley agar with and without 0.05% w/v sodium pyruvate supplementation was used. Azide dextrose broth tubes and unsupplemented Slanetz and Bartley agar plates were incubated under standard aerobic conditions, while plates supplemented with pyruvate were incubated under anaerobic conditions at an

incubation temperature of 37°C for 48 h. Confirmation of turbid azide dextrose tubes and typical red-maroon colonies from Slanetz and Bartley agar (presumptive enterococci) was by streaking on aesculin agar (Labchem Himedia, Mumbai, India) which confirmed as *Enterococcus* spp. if they formed black coloured halo-zoned colonies. Statistical analysis was carried out using the Wilcoxon (matched pairs) signed rank test described above.

5.3 Results

5.3.1 Comparison of resuscitation media (total coliforms) and pre-enrichment (enterococci) for the enumeration of faecal indicator bacteria in water samples

Total coliforms Figure 5.1a provides data for 50 water samples processed to obtain a presumptive count for total coliforms under aerobic conditions. In this figure each data point represents a single water sample analysed using two different growth media, the X-axis is for the resuscitation medium m-T7 agar and the Y-axis is for MacConkey agar, with both expressed as counts in CFU 100 mL^{-1}. Note that the X-axis and Y-axis are log-transformed. The data point for each sample is the value obtained on each growth medium and in all subsequent results such data will be represented in this format as "paired counts". The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. If the points lie on the dotted line or close to it then the paired counts of both media are equal in magnitude whereas if most of the points lie towards the X-axis or the Y-axis then one medium is consistently showing higher counts than the other medium. This is the recommended approach for method comparison in UK standard methods (Report 71; Anon., 2002). In Fig. 5.1a, comparing both media it appeared that 29 out of 50 paired counts of water samples gave higher counts on m-T7 agar; one out of 50 gave a similar count for both media, while 20 out of 50 gave higher counts on MacConkey agar. Thus under standard aerobic conditions m-T7 agar (mean count = 2825 CFU mL^{-1}) performed better than MacConkey agar (mean count= 2546 CFU mL^{-1}). The Wilcoxon sign rank test showed a statistically significant difference between the two growth media as the calculated T value was less than the T critical value at a *P* value of 0.05 (see Table 5.1).

Figure 5.1b-d shows paired counts for total coliforms enumerated on resuscitation medium m-T7 agar (X-axis), compared with mFC agar (Fig 5.1b), mEndo agar (Fig. 5.1c) and mLSA agar (Fig. 5.1d), all plotted on the Y-axis. For all three cases m-T7 agar recovered on average a statistically significantly higher number of coliforms ($P<0.01$) compared with these selective media (Table 5.1).

Figure 5.2a-d represents total coliform data obtained under ROS-neutralised conditions (pyruvate supplemented medium and anaerobic growth conditions) for paired plate counts enumerated on m-T7 agar (X-axis) compared with other selective media, i.e. MacConkey agar (Fig. 5.2a), mFC agar (Fig 5.2b), mEndo agar (Fig. 5.2c) and mLSA agar (Fig. 5.2d), all plotted on the Y-axis. The results under ROS-neutralised conditions showed no statistically significant difference ($P>0.05$) between the count enumerated by m-T7 agar and MacConkey agar (Fig. 5.2a; Table 5.1) but showed statistically significant difference ($P<0.01$) between m-T7 agar and all other selective media (Fig. 5.2b-d; Table 5.1).

When aerobic and ROS-neutralised paired counts for the resuscitative medium m-T7 agar were compared (Fig. 5.3), a statistically significant difference between the two growth conditions was obtained ($P<0.01$; Table 5.1) with a mean count under ROS-neutralised conditions approximately double that of the aerobic value. Thus pyruvate supplemented m-T7 medium incubated under ROS-neutralised conditions proved to enhance the counts obtained using this resuscitative medium.

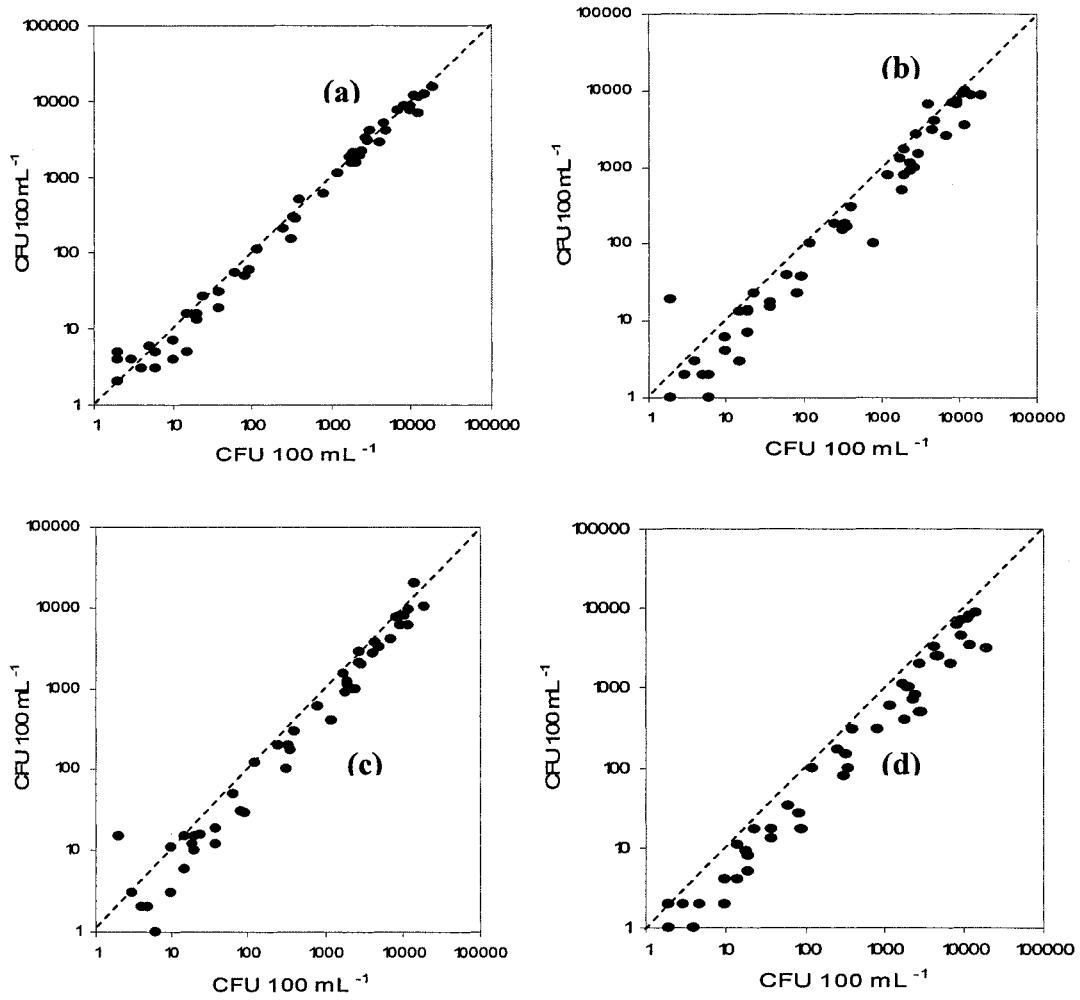


Fig. 5.1 Comparison of a resuscitation medium and selective media under aerobic conditions for the enumeration of total coliforms from water samples with each data point represented for a single water sample analysed using two different growth media, i.e. (a) m-T7 agar (X-axis) and MacConkey agar (Y-axis), (b) m-T7 agar (X-axis) and mFC-R agar (Y-axis), (c) m-T7 agar (X-axis) and mEndo agar (Y-axis), (d) m-T7 agar (X-axis) and mLSA agar (Y-axis). The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

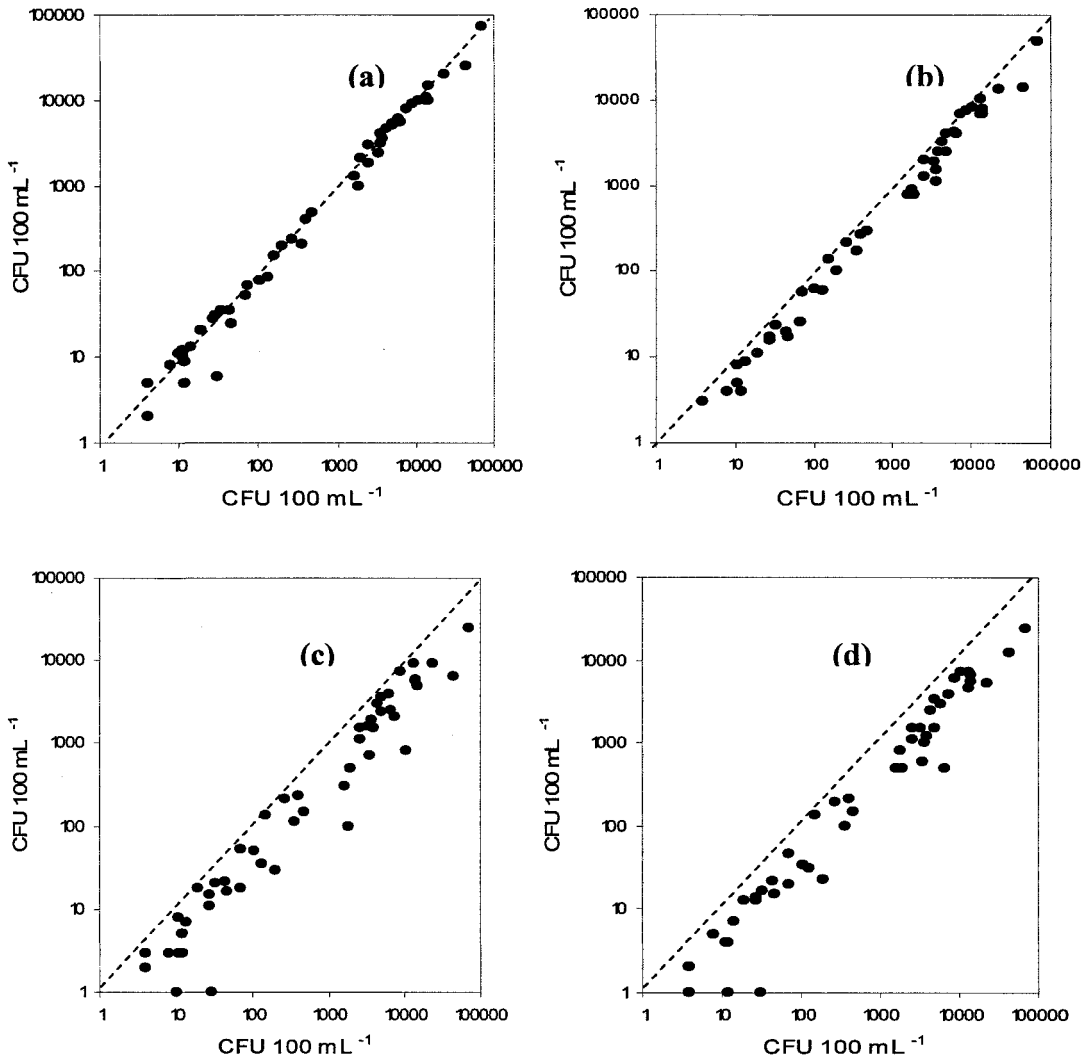


Fig. 5.2 Comparison of resuscitation medium and selective media under ROS-neutralised conditions for the enumeration of total coliforms from water samples with each data point represented for a single water sample analysed using two different growth media, i.e. (a) m-T7 agar (X-axis) and MacConkey agar (Y-axis), (b) m-T7 agar (X-axis) and mFC-R agar (Y-axis), (c) m-T7 agar (X-axis) and mEndo agar (Y-axis), (d) m-T7 agar (X-axis) and mLSA agar (Y-axis). The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

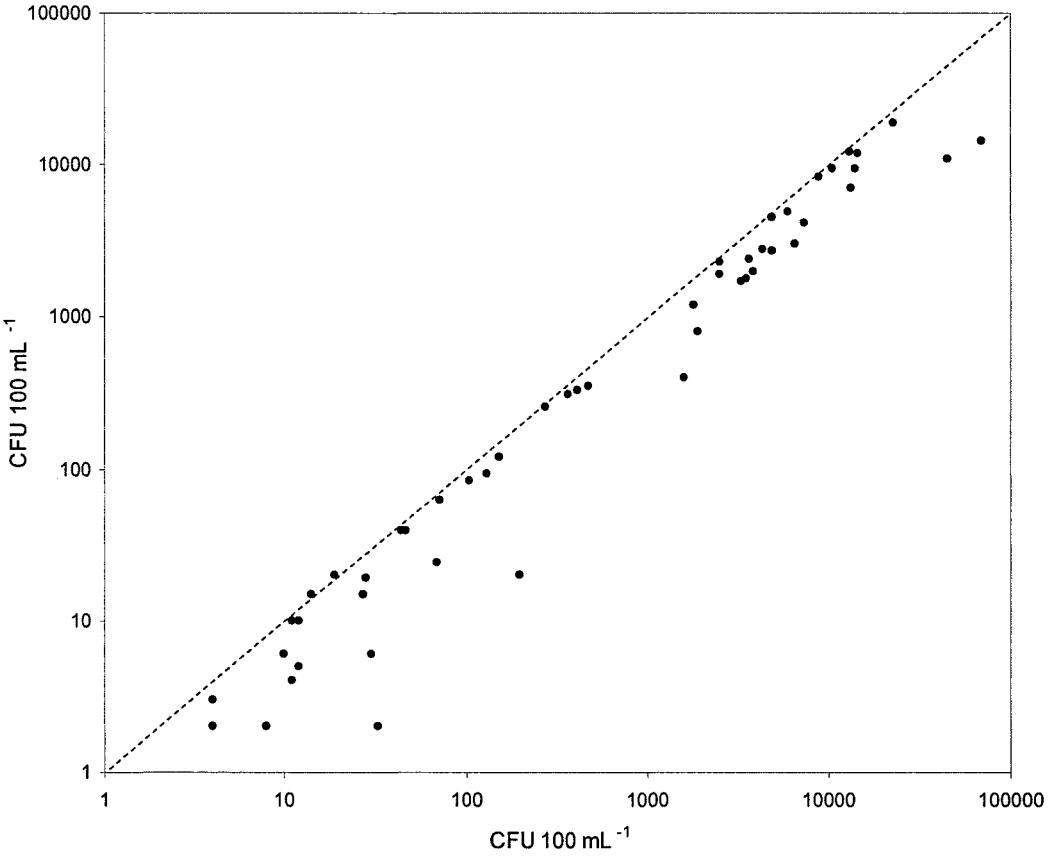


Fig. 5.3 Comparison of resuscitation medium under aerobic and ROS-neutralised conditions for the enumeration of total coliforms from water samples with each data point represented for a single water sample analysed using two different sets of growth conditions, i.e. m-T7 agar incubated under ROS-neutralised conditions (X-axis) and m-T7 agar incubated under aerobic conditions (Y-axis). The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

Table 5.1 Comparison of a resuscitation medium and various selective media under aerobic and ROS-neutralised conditions for the enumeration of total coliforms from 50 water samples using the non - parametric Wilcoxon matched pairs signed rank test

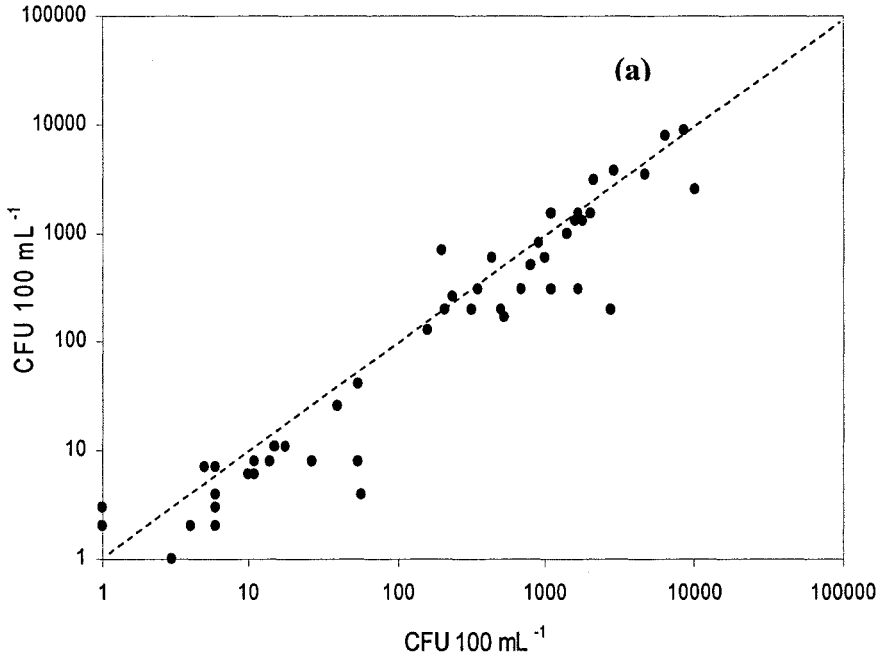
Comparisons [(1) and (2)]	Mean (1)	Mean (2)	T	N	C (0.05)	C (0.01)	P
(1) mT7 (aer) and (2) Mac (aer)	2825	2546	386	49	415	356	<0.05
(1) mT7 (aer) and (2) mFC-R (aer)	2825	1799	21	50	434	373	<0.01
(1) mT7 (aer) and (2) mEndo (aer)	2825	2117	82	47	397	323	<0.01
(1) mT7 (aer) and (2) mLSA (aer)	2825	1379	0*	49	415	356	<0.01
(1) mT7 (ROS^n) and (2) Mac (ROS^n)	5463	4879	458	49	415	356	>0.05
(1) mT7 (ROS^n) and (2) mFC-R (ROS^n)	5463	3301	0*	50	434	373	<0.01
(1) mT7 (ROS^n) and (2) mEndo	5463	2055	0*	50	434	373	<0.01
(1) mT7 (ROS^n) and (2) mLSA	5463	2049	0*	50	434	373	<0.01
(1) mT7 (aer) and (2) mT7(ROS^n)	2825	5463	5	50	434	373	<0.01

mT7 = mT7 agar (resuscitative medium)
Mac = MacConkey agar (selective medium)
mFC-R = membrane coliform agar without rosolic acid (selective medium)
mEndo = mEndo agar (selective medium)
mLSA = membrane lauryl sulphate agar as a selective medium
aer = aerobic conditions
ROS^n = ROS-neutralised conditions
T = smaller of T+ and T- from Wilcoxon Signed Rank Test
N = number of non-zero differences
C (0.05) = critical value for the Wilcoxon matched pairs signed rank test at two-sided *P* value of 0.05 and at the specified value of N (Kirkwood *et al.*, 2003)
C (0.01) = critical value for the Wilcoxon matched pairs signed rank test at two-sided *P* value of 0.01 and at the specified value of N (Kirkwood *et al.*, 2003)
P = two sided probability, based on the comparison of T and C values
* Note: all values for these comparisons were higher for medium 1

Enterococci paired counts for 50 water samples are shown in Fig. 5.4 obtained under aerobic conditions (Fig. 5.4a) and ROS-neutralised conditions (Fig. 5.4b), enumerated on Slanetz and Bartley agar with (X-axis) and without (Y-axis) inclusion of a 3-hour pre-enrichment step. The results for Slanetz and Bartley agar with and without inclusion of a pre-enrichment step under aerobic conditions showed a statistically highly significant difference ($P < 0.01$), while under ROS-neutralised conditions, Slanetz and Bartley agar with and without the inclusion of pre-enrichment step showed no statistically significant difference ($P > 0.05$) between the two conditions, with very similar mean values for both (Table 5.2). Thus by enumerating under ROS-neutralised conditions the pre-enrichment step might be excluded for this medium with no substantial reduction in count, unlike under aerobic conditions (c.f. Fig. 5.4a).

Fig. 5.5 provides results for a similar experiment carried out using KF streptococcus agar instead of Slanetz and Bartley agar, with and without the inclusion of a pre-enrichment step, with incubation under standard aerobic conditions (Fig. 5.5a) and ROS-neutralised conditions (Fig. 5.5b). A highly significant different was observed when the pre-enrichment step was included under aerobic conditions ($P < 0.01$) but not under ROS-neutralised conditions ($P > 0.05$; Table 5.2).

Fig. 5.6 represents results under aerobic conditions (Fig. 5.6a) and ROS-neutralised conditions (Fig. 5.6b) obtained for enterococci after comparing paired counts using Slanetz and Bartley agar (Y-axis) and KF streptococcus agar (X-axis) with the inclusion of a bile broth pre-enrichment step for both media. The statistical analysis of the counts obtained for both media showed no significant difference ($P > 0.05$ for both comparisons) after the pre-enrichment step (Table 5.2).



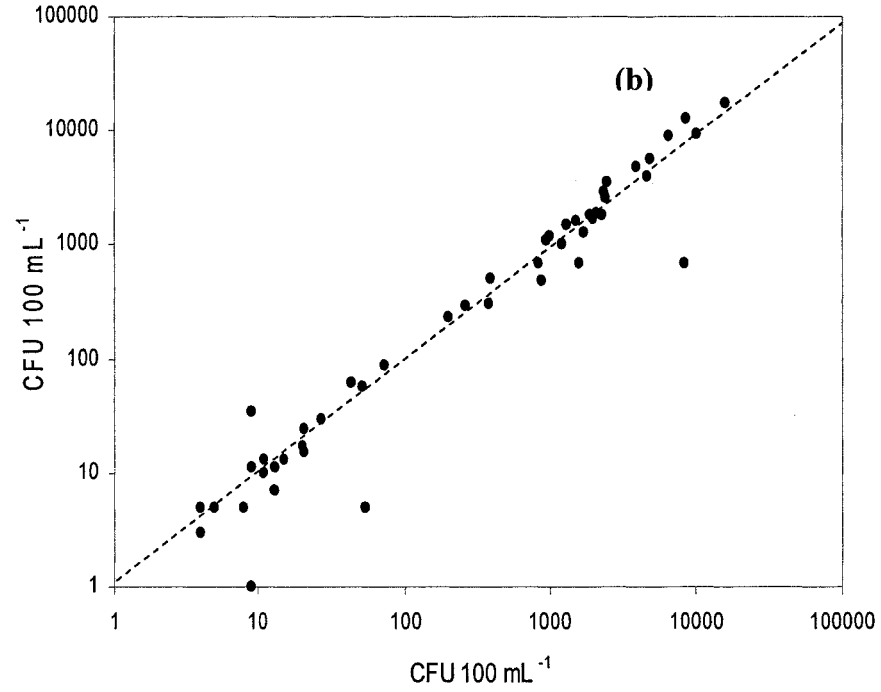


Fig. 5.4 Comparisons of a pre-enrichment step under aerobic and ROS-neutralised conditions for the enumeration of enterococci from water samples with each data point represented for a single water sample analysed using two different growth media, i.e. (a) Slanetz and Bartley agar with pre-enrichment on bile broth (X-axis) and Slanetz and Bartley agar without pre-enrichment (Y-axis) both under aerobic conditions, (b) Slanetz and Bartley agar with pre-enrichment on bile broth (X-axis) and Slanetz and Bartley agar without pre-enrichment (Y-axis) both under ROS-neutralised conditions. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

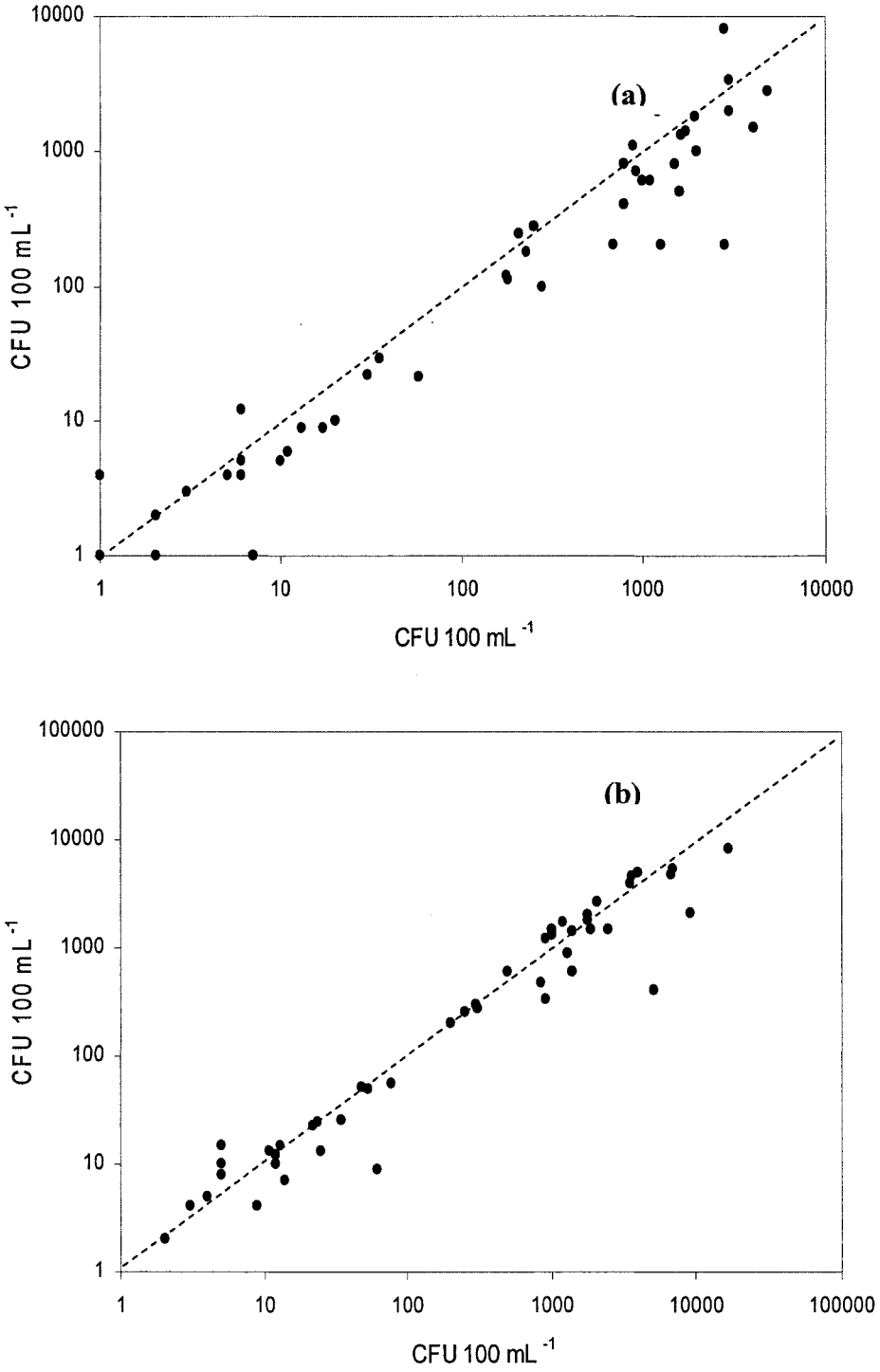


Fig. 5.5 Comparisons of a pre-enrichment step under aerobic and ROS-neutralised conditions for the enumeration of enterococci from water samples with each data point represented for a single water sample analysed using two different growth media, i.e. (a) KF streptococcus agar with pre-enrichment on bile broth (X-axis) and KF streptococcus agar without pre-enrichment (Y-axis) both under aerobic conditions, (b) KF streptococcus agar with pre-enrichment on bile broth (X-axis) and KF streptococcus agar without pre-enrichment (Y-axis) both under ROS-neutralised conditions. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

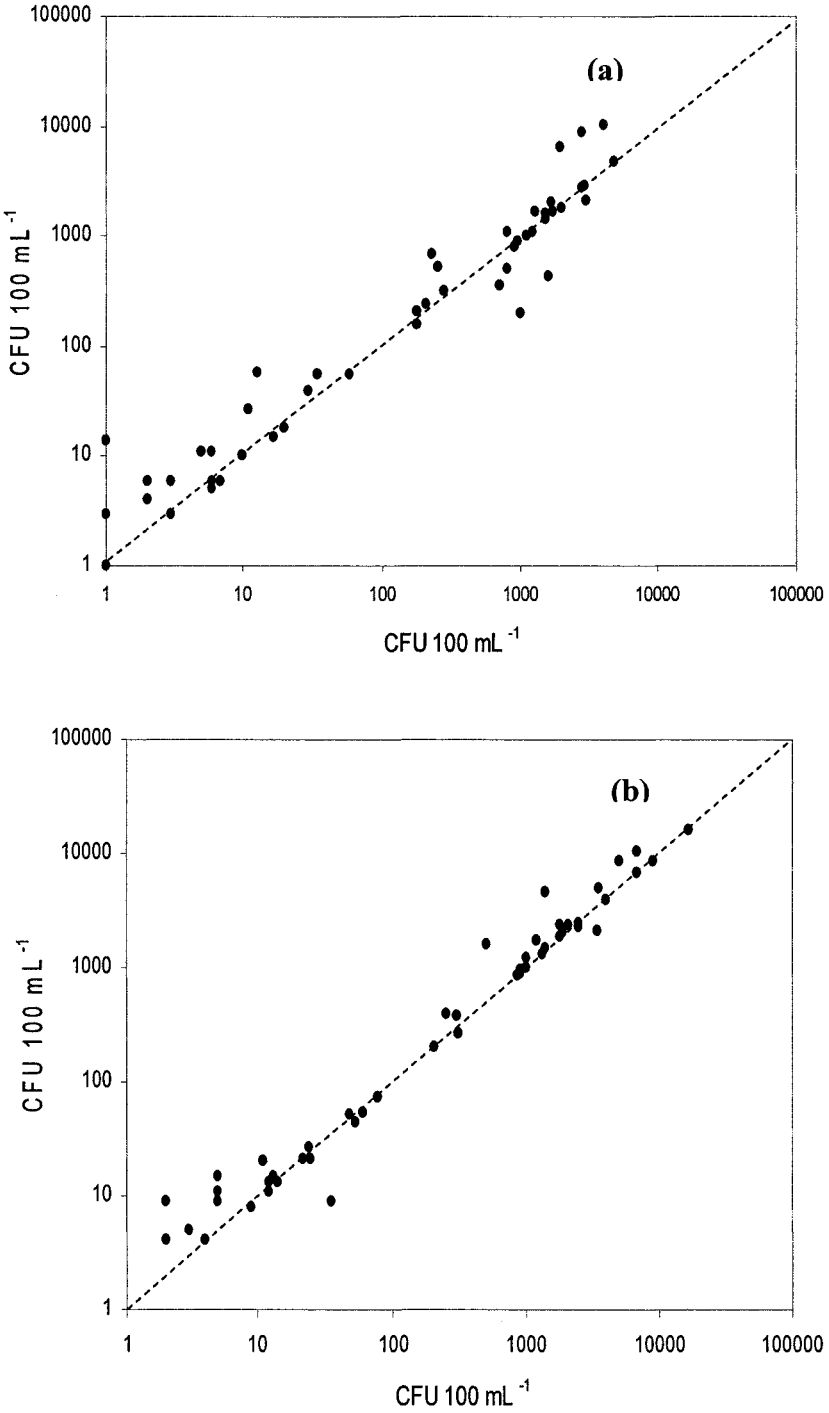


Fig. 5.6 Comparisons of a pre-enrichment step under aerobic and ROS-neutralised conditions for the enumeration of enterococci from water samples with each data point represented for a single water sample analysed using two different growth media, i.e. (a) KF streptococcus agar with pre-enrichment on bile broth (X-axis) and Slanetz and Bartley agar with pre-enrichment (Y-axis) both under aerobic conditions, (b) KF streptococcus agar with pre-enrichment on bile broth (X-axis) and Slanetz and Bartley agar with pre-enrichment (Y-axis) both under ROS-neutralised conditions. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

Table 5.2 Comparisons of selective media with and without a pre-enrichment step under aerobic and ROS - neutralised conditions for the enumeration of enterococci from 50 water samples using the non - parametric Wilcoxon matched pairs signed rank test

Comparisons [(1) and (2)]	Mean (1)	Mean (2)	T	N	C (0.05)	C (0.01)	*P*
(1) SB pre (aer) and (2) SB (aer)	1140	873	304	50	434	373	<0.01
(1) SB pre (ROS) and (2) SB (ROS^n)	1841	1819	582	49	415	356	>0.05
(1) KF pre (aer) and (2) KF (aer)	856	666	216	45	344	221	<0.01
(1) KF pre (ROS^n) and (2) KF (ROS^n)	1607	1134	475	45	344	221	>0.05
(1) SB pre (aer)and (2) KF pre (aer)	1140	856	460	44	327	277	>0.05
(1) SB pre (ROS^n) and (2) KF pre (ROS^n)	1841	1607	392	46	389	307	>0.05

SB pre = Slanetz and Bartley agar with inclusion of pre-enrichment step
SB = Slanetz and Bartley agar without inclusion of pre-enrichment step
KF pre = KF streptococcus agar with inclusion of pre-enrichment step
KF = KF streptococcus agar without inclusion of pre-enrichment step
aer = aerobic conditions
ROS^n = ROS-neutralised conditions
T = smaller of T+ and T- from Wilcoxon Signed Rank Test
N = number of non-zero differences
C (0.05) = critical value for the Wilcoxon matched pairs signed rank test at two-sided *P* value of 0.05 and at the specified value of N (Kirkwood *et al.*, 2003)
C (0.01) = critical value for the Wilcoxon matched pairs signed rank test at two-sided *P* value of 0.01 and at the specified value of N (Kirkwood *et al.*, 2003)
P = two sided probability, based on the comparison of T and C values

5.3.2 Comparisons of standard most probable number (MPN) technique and membrane filtration (MF) technique for the enumeration of faecal indicator bacteria in water samples

Escherichia coli Fig. 5.7a shows presumptive MPN results obtained for 50 water samples enumerating under standard aerobic conditions using MacConkey broth (X-axis) as MPN 100 mL $^{-1}$ and MacConkey agar (Y-axis) as CFU 100 mL $^{-1}$. Thus 63% of the samples gave a higher value by MacConkey broth by the MPN method compared to the corresponding value for MacConkey agar by the MF method. The confirmed results for *Escherichia coli* from the presumptive data (from Fig. 5.7a) are shown in Fig. 5.7b. The confirmed results show a statistically highly significant difference between the performances of both of the techniques at a *P* value of 0.01, with the mean value approximately 50% higher by MF under aerobic conditions (Table 5.3)

Presumptive results are shown in Fig. 5.8a for the MPN method using MacConkey broth (X-axis) and the MF method using MacConkey agar incubated under ROS-neutralised conditions (Y-axis). Thus 66% of samples gave a higher count using ROS-neutralised conditions for MacConkey medium (MF method) when compared to MacConkey broth (MPN method). The presumptive results (Fig. 5.8a) after confirmation for *Escherichia coli* (Fig. 5.8b) showed a statistically significant difference between the two techniques at a *P* value of 0.01, with the mean by MF method more than double than MPN method (Table 5.3).

Fig. 5.9a provides results for presumptive count using the MF technique with enumeration under standard aerobic conditions (X-axis), or ROS-neutralised conditions (Y-axis). Thus 84% of the samples gave a higher count on ROS-

neutralised MacConkey agar compared to the aerobic counterpart. After confirmation (Fig. 5.9b) of the presumptive count a statistically significant difference between the two sets of data was obtained at a *P* value of 0.01, with the MF count under ROS-neutralized conditions being almost 50% higher than the aerobic value (Table 5.3).

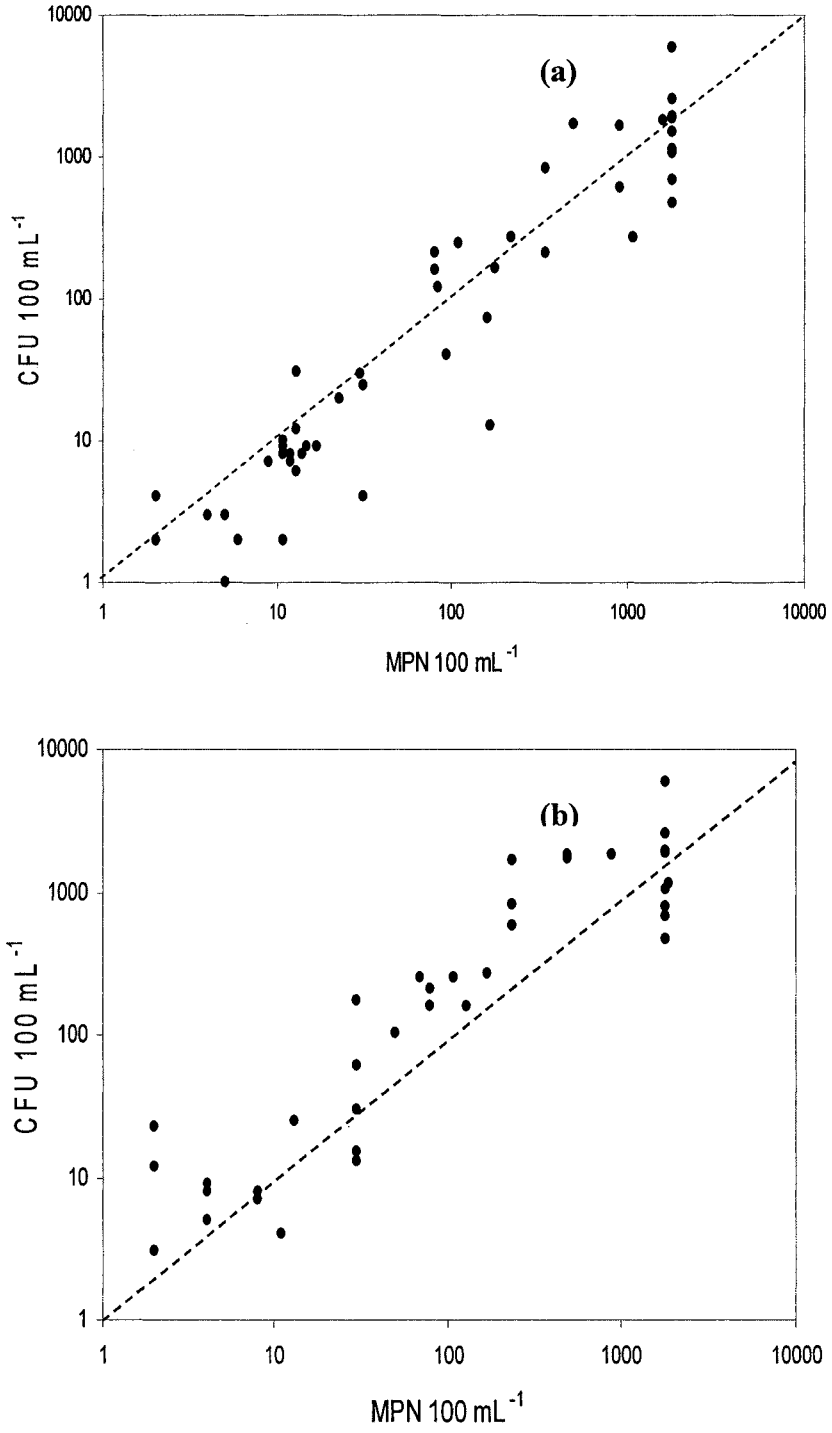


Fig. 5.7 Comparisons of MPN and MF techniques for the enumeration of *Escherichia coli* in water samples with each data point represented for a single water sample analysed using two different methods enumerated under standard aerobic conditions, i.e. (a) presumptive count for MacConkey broth in MPN method plotted on X-axis (MPN 100 mL $^{-1}$) and MacConkey agar in MF method plotted on Y-axis (CFU 100 mL $^{-1}$), (b) confirmed count for MacConkey broth in MPN method plotted on X-axis and MacConkey agar in MF method plotted on Y-axis. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

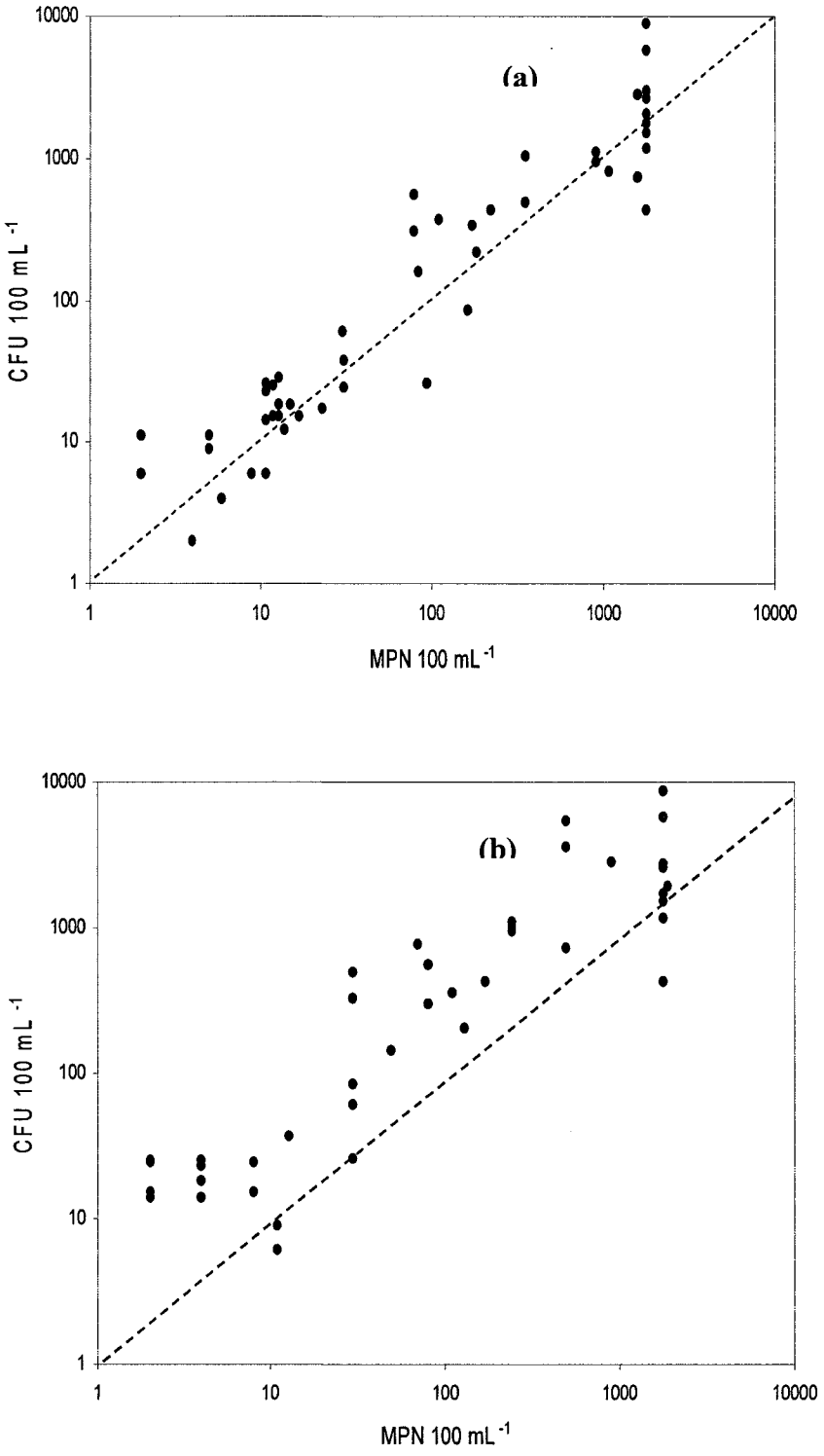


Fig. 5.8 Comparisons of MPN and MF techniques for the enumeration of *Escherichia coli* in water samples with each data point represented for a single water sample analysed using two different methods, i.e. (a) presumptive count for MacConkey broth in MPN method plotted on X-axis (MPN 100 mL $^{-1}$) and MacConkey agar incubated under ROS-neutralised conditions in MF method plotted on Y-axis (CFU 100 mL $^{-1}$), (b) confirmed count for MacConkey broth in MPN method plotted on X-axis and MacConkey agar incubated under ROS-neutralised conditions in MF method plotted on Y-axis. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

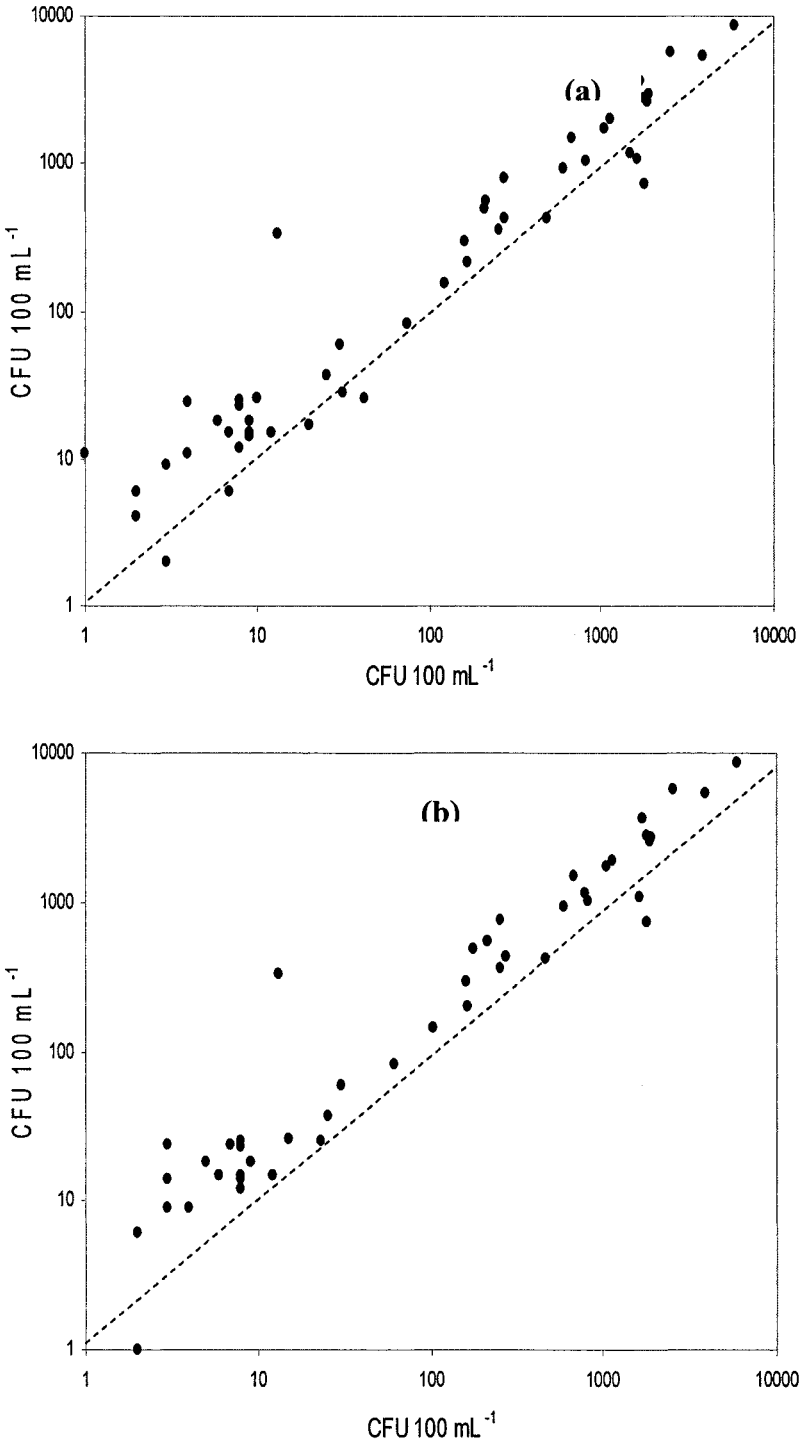


Fig. 5.9 Comparisons of two growth conditions (aerobic and ROS-neutralised) using MF assays for the enumeration of *Escherichia coli* in water samples with each data point represented for a single water sample analysed using MF method enumerated under two sets of growth conditions, i.e. (a) presumptive count for MacConkey agar incubated under aerobic conditions on X-axis and MacConkey agar incubated under ROS-neutralised conditions on Y-axis, (b) confirmed count for MacConkey agar incubated under aerobic conditions on X-axis and MacConkey agar incubated under ROS-neutralised conditions on Y-axis. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

Table 5.3 Comparisons of confirmed values obtained by using MPN and MF techniques under aerobic and ROS-neutralised conditions for the enumeration of *Escherichia coli* from 50 water samples using the non - parametric Wilcoxon matched pairs signed rank test

Comparisons [(1) and (2)]	Mean (1)	Mean (2)	T	N	C (0.05)	C (0.01)	P
(1) MPN (aer) and (2) MF (aer)	407	610	204	46	361	307	<0.01
(1) MPN (aer) and (2) MF (ROS^n)	407	920	130	49	415	356	<0.01
(1) MF (aer) and (2) MF (ROS^n)	610	920	103	48	397	339	<0.01

MPN = most probable number technique using MacConkey broth
MF = membrane filtration technique using MacConkey agar
aer = aerobic conditions
ROS^n = ROS-neutralised conditions
T = smaller of T+ and T- from Wilcoxon Signed Rank Test
N = number of non-zero differences
C (0.05) = critical value for the Wilcoxon matched pairs signed rank test at two-sided *P* value of 0.05 and at the specified value of N (Kirkwood *et al.*, 2003)
C (0.01) = critical value for the Wilcoxon matched pairs signed rank test at two-sided *P* value of 0.01 and at the specified value of N (Kirkwood *et al.*, 2003)
P = two sided probability, based on the comparison of T and C values

Fig. 5.10a-c describes results for paired comparisons made between presumptive values (X-axis) and confirmed values (Y-axis) for the MPN using MacConkey broth (Fig. 5.10a), MF-aerobic MacConkey agar (Fig. 5.10b), and MF-ROS-neutralised MacConkey agar (Fig. 5.10c). The results depict that maximum false positives were obtained using the MPN method with 70% of presumptive values giving false positive results when confirmed as shown in Fig. 5.10a, while 42% of presumptive values giving false positives when confirmed for MF-aerobic medium in Fig. 5.10b and only 28% of presumptive values giving false positives when confirmed for MF-ROS-neutralised medium in Fig. 5.10c, which also clearly shows the very close agreement between presumptive and confirmed values.

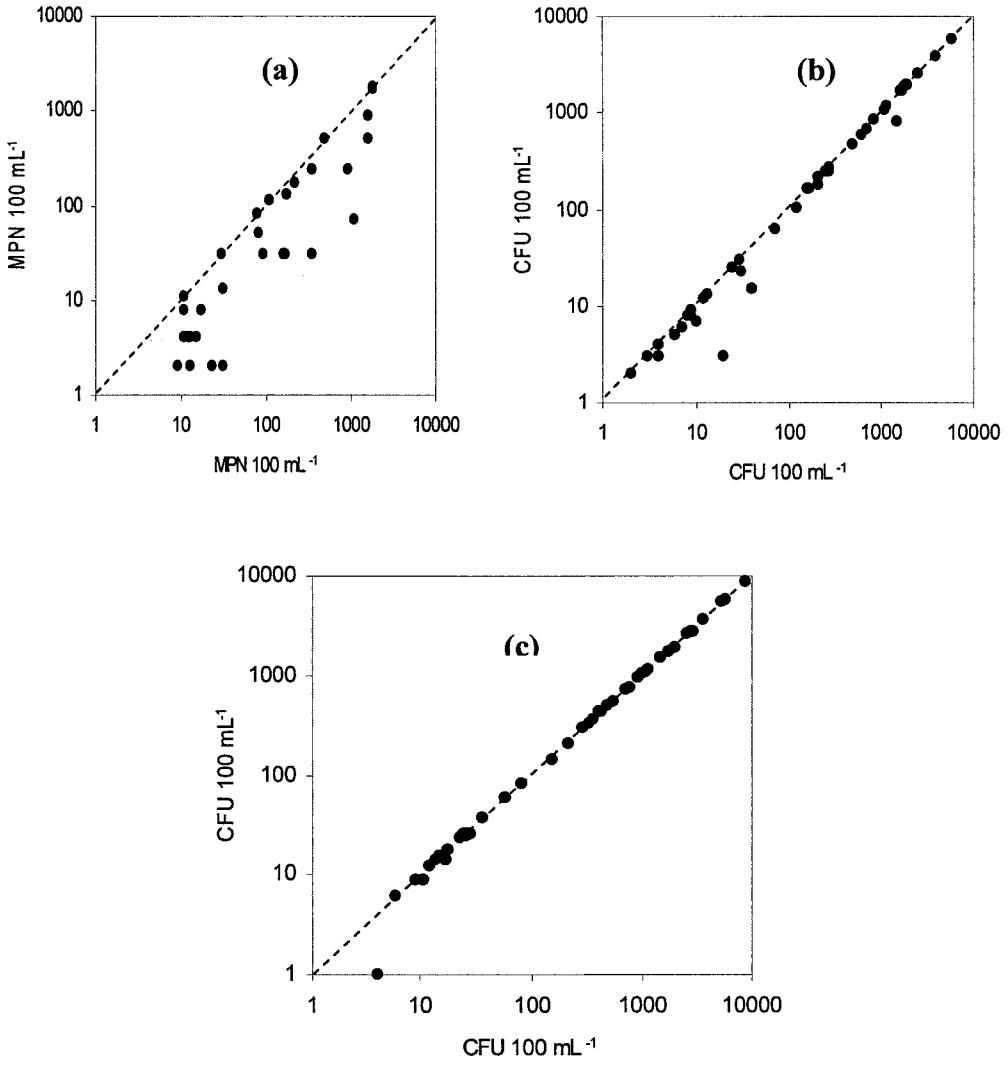


Fig. 5.10 Comparisons of presumptive and confirmed values using MPN and MF techniques for the enumeration of *Escherichia coli* in water samples with each data point represented for a single water sample analysed using MPN or MF method, i.e. (a) presumptive count for MacConkey broth (X-axis) and confirmed count for MacConkey broth (Y-axis) using MPN method, (b) presumptive count for MacConkey agar (X-axis) and confirmed count for MacConkey agar (Y-axis) under aerobic conditions using MF method, (c) presumptive count for MacConkey agar (X-axis) and confirmed count for MacConkey agar (Y-axis) under ROS-neutralised conditions using MF method. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

***Enterococcus* spp.** Presumptive MPN results for 50 water samples enumerated for enterococci are shown in Figure 5.11a, under standard aerobic conditions using azide dextrose broth in an MPN assay (X-axis) against Slanetz and Bartley agar in an MF assay (Y-axis). The presumptive values gave higher results in 60% of samples by the MPN assay (azide dextrose broth) compared to corresponding values obtained from MF assay (Slanetz and Bartley agar). After confirmation, a statistically significant difference between the two sets of data was obtained at a *P* value of 0.01, with the MF method giving a higher overall mean value (Fig. 5.11b; Table 5.4).

Fig. 5.12a shows results for MPN-azide dextrose broth (X-axis) and MF-ROS-neutralised Slanetz and Bartley medium (Y-axis). The presumptive results show both methods to be broadly equivalent for the enumeration of enterococci. The confirmed results for both assays showed a significant difference at a *P* value of 0.01, with the MF method giving a mean value of more than double than average MPN value (Fig. 5.12b; Table 5.4).

Fig. 5.13a provides results for presumptive enterococci using Slanetz and Bartley agar with enumeration by MF technique under two sets of growth conditions, i.e. standard aerobic conditions (X-axis) and ROS-neutralised conditions (Y-axis). Thus results indicate that ROS-neutralised Slanetz and Bartley medium recover more enterococci than its counterpart aerobic medium. The confirmed results from presumptive counts on Slanetz and Bartley medium for aerobic and ROS-neutralised medium showed a statistically significant differences between the two sets of enumeration conditions at a *P* value of 0.01 with the mean of ROS-neutralised count being almost double the average aerobic value (Fig. 5.13b; Table 5.4).

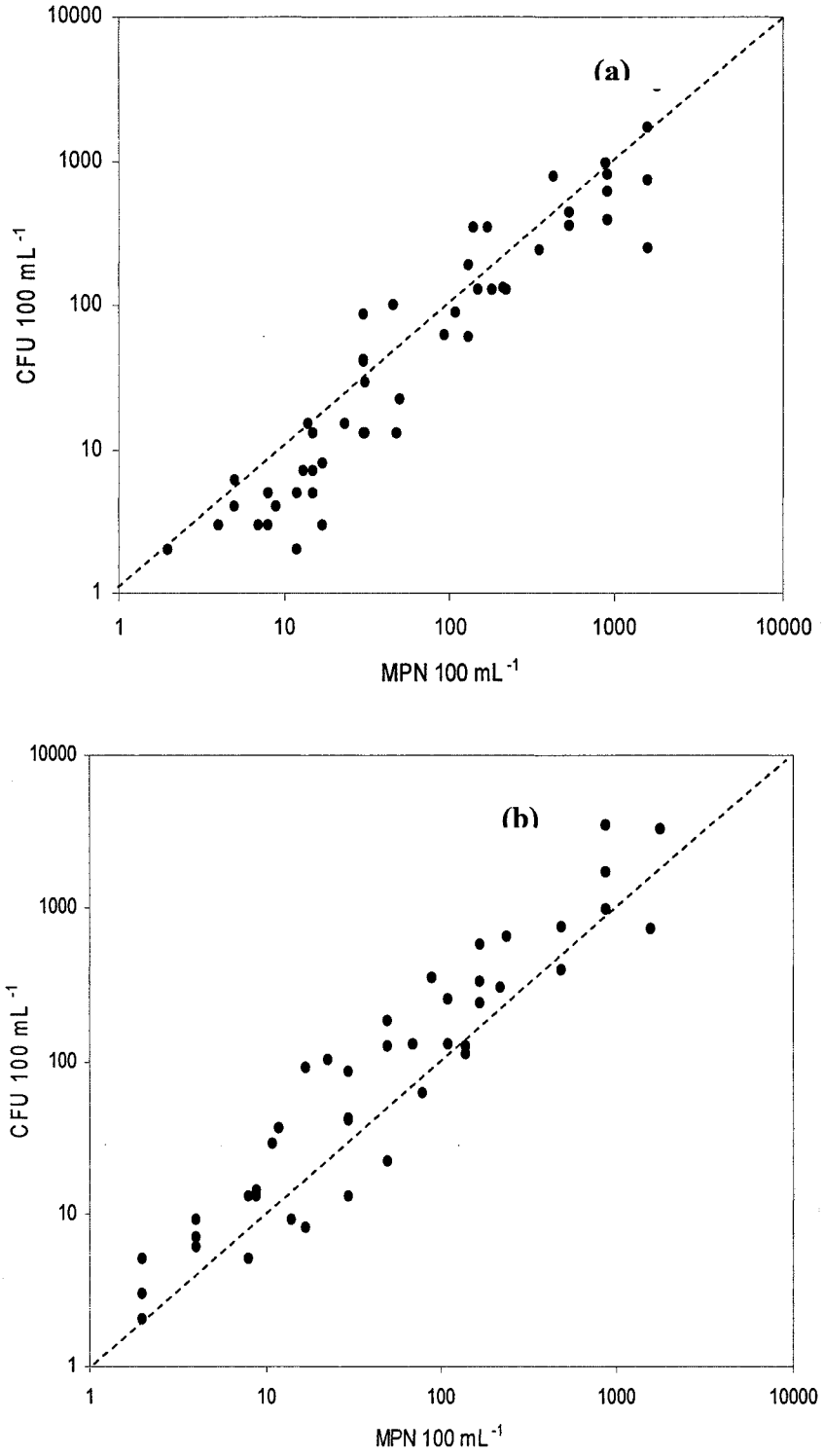


Fig. 5.11 Comparisons of MPN and MF techniques for the enumeration of *Enterococcus spp.* in water samples with each data point represented for a single water sample analysed using two different methods enumerated under standard aerobic conditions, i.e. (a) presumptive count for azide dextrose broth in MPN method plotted on X-axis (MPN 100 mL $^{-1}$) and Slanetz and Bartley agar in MF method plotted on Y-axis (CFU 100 mL $^{-1}$), (b) confirmed count for azide dextrose broth in MPN method plotted on X-axis and Slanetz and Bartley agar in MF method plotted on Y-axis. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

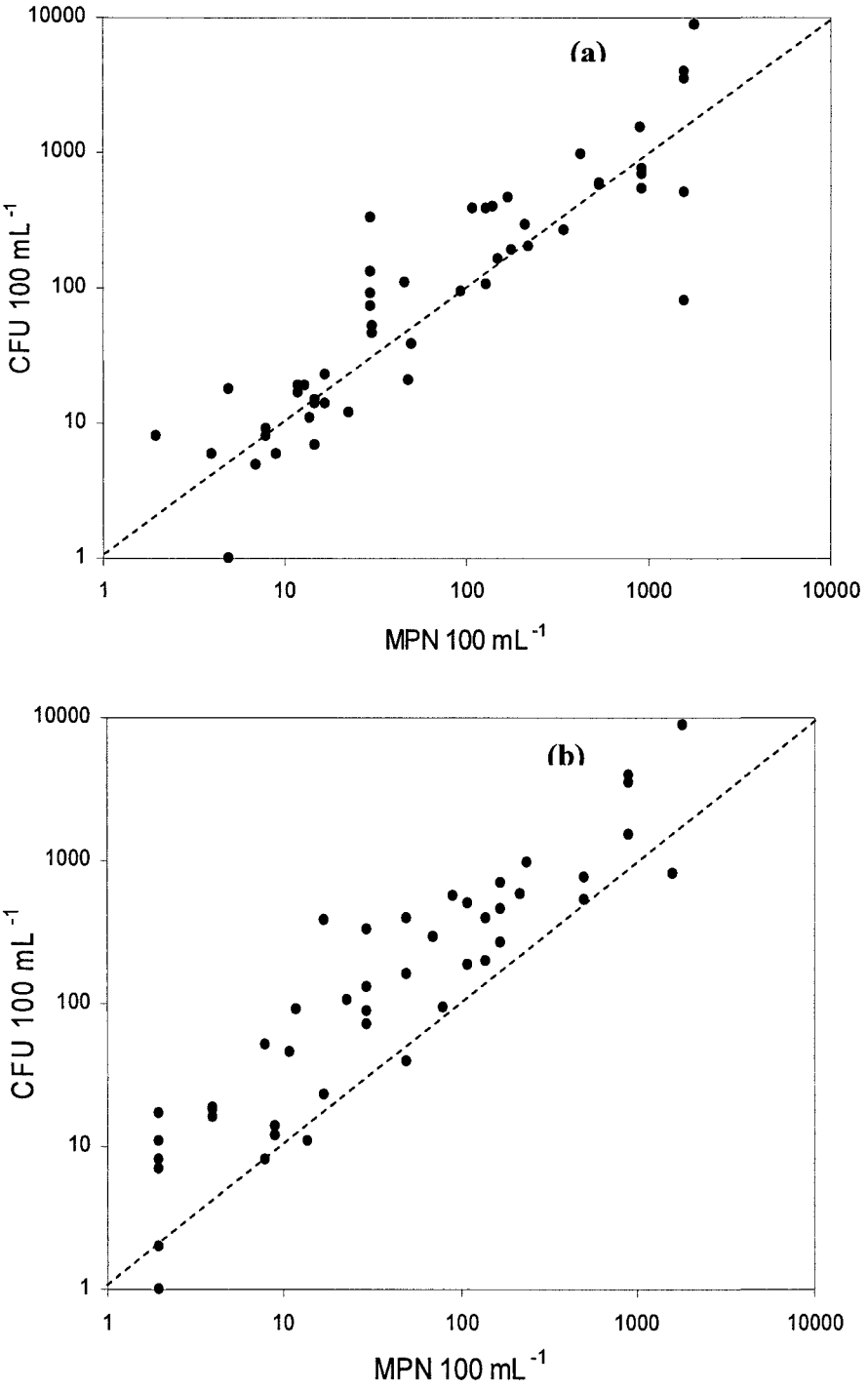


Fig. 5.12 Comparisons of MPN and MF techniques for the enumeration of *Enterococcus spp.* in water samples with each data point represented for a single water sample analysed using two different methods, i.e. (a) presumptive count for azide dextrose broth in MPN method plotted on X-axis (MPN 100 mL $^{-1}$) and Slanetz and Bartley agar incubated under ROS-neutralised conditions in MF method plotted on Y-axis (CFU 100 mL $^{-1}$), (b) confirmed count for azide dextrose broth in MPN method plotted on X-axis and Slanetz and Bartley agar incubated under ROS-neutralised conditions in MF method plotted on Y-axis. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

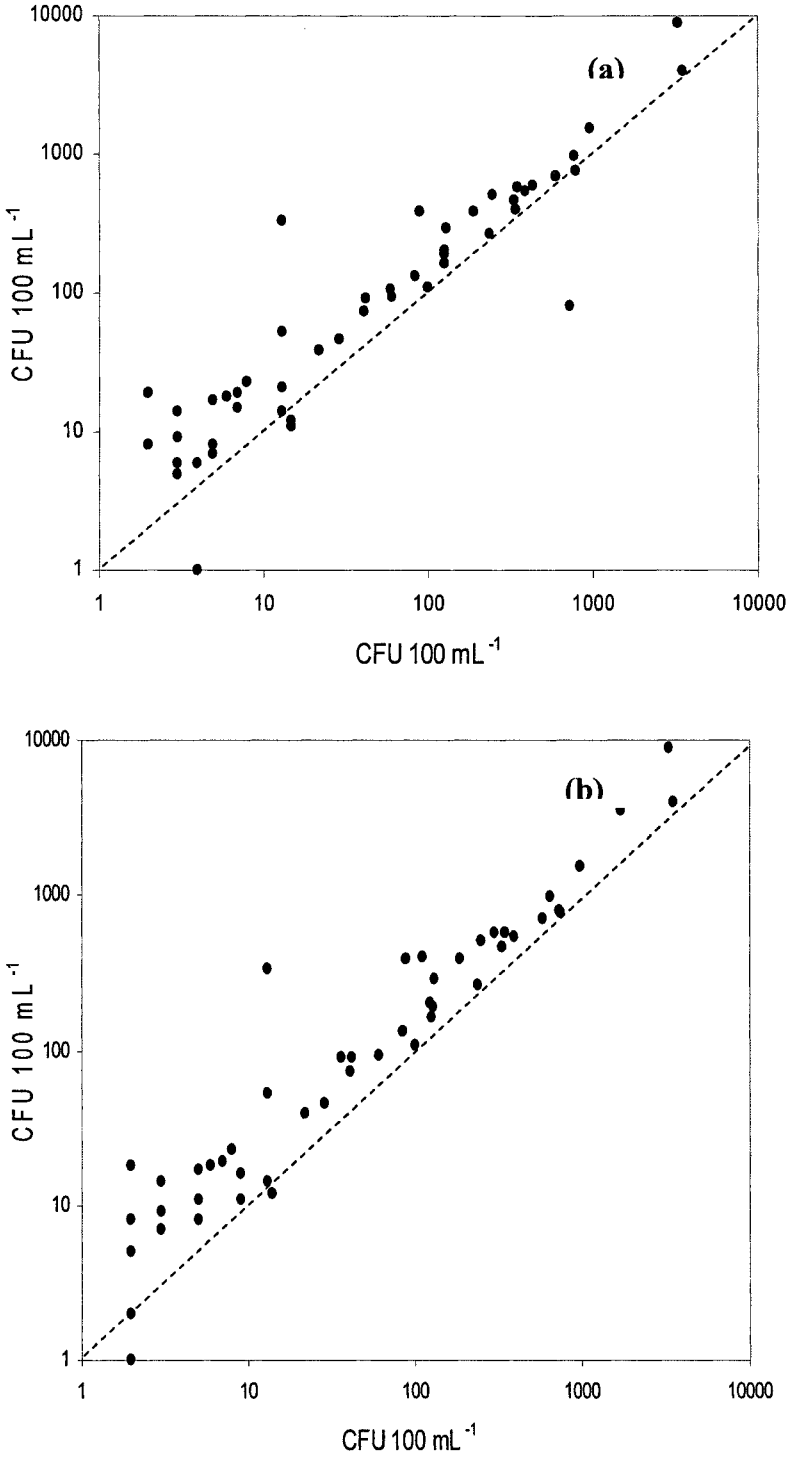


Fig. 5.13 Comparisons of two growth conditions (aerobic and ROS-neutralised) using MF assays for the enumeration of *Enterococcus spp.* in water samples with each data point represented for a single water sample analysed using MF method enumerated under two sets of growth conditions, i.e. (a) presumptive count for Slanetz and Bartley agar incubated under aerobic conditions on X-axis and Slanetz and Bartley agar incubated under ROS-neutralised conditions on Y-axis, (b) confirmed count for Slanetz and Bartley agar incubated under aerobic conditions on X-axis and Slanetz and Bartley agar incubated under ROS-neutralised conditions on Y-axis. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

Table 5.4 Comparisons of confirmed values obtained by using MPN and MF techniques under aerobic and ROS-neutralised conditions for the enumeration of *Enterococcus spp.* from 50 water samples using the non - parametric Wilcoxon matched pairs signed rank test

Comparisons [(1) and (2)]	Mean (1)	Mean (2)	T	N	C (0.05)	C (0.01)	P
(1) MPN (aer) and (2) MF (aer)	184	310	258	48	397	339	<0.01
(1) MPN (aer) and (2) MF (ROS^n)	184	548	62	48	397	339	<0.01
(1) MF (aer) and (2) MF (ROS^n)	310	548	7	49	415	356	<0.01

MPN = most probable number technique using azide dextrose broth
MF = membrane filtration technique using Slanetz and Bartley agar
aer = aerobic conditions
ROS^n = ROS-neutralised conditions
T = smaller of T+ and T- from Wilcoxon Signed Rank Test
N = number of non-zero differences
C (0.05) = critical value for the Wilcoxon matched pairs signed rank test at two-sided *P* value of 0.05 and at the specified value of N (Kirkwood *et al.*, 2003)
C (0.01) = critical value for the Wilcoxon matched pairs signed rank test at two-sided *P* value of 0.01 and at the specified value of N (Kirkwood *et al.*, 2003)
P = two sided probability, based on the comparison of T and C values

Comparisons between presumptive values (X-axis) and confirmed values (Y-axis) are shown for azide dextrose broth (MPN method) in Fig. 5.14a, Slanetz and Bartley agar (MF method) for aerobic conditions in Fig. 5.14b, and Slanetz and Bartley agar(MF method) for ROS-neutralised conditions in Fig. 5.14c. The results depict that maximum false positives were obtained using the MPN method with 72% of presumptive values giving false positive results when confirmed as shown in Fig. 5.14a, with only 36% confirming as calculated for aerobic Slanetz and Bartley medium (Fig. 5.14b) and only 24% false-negatives for ROS-neutralised Slanetz and Bartley medium (Fig. 5.14c).

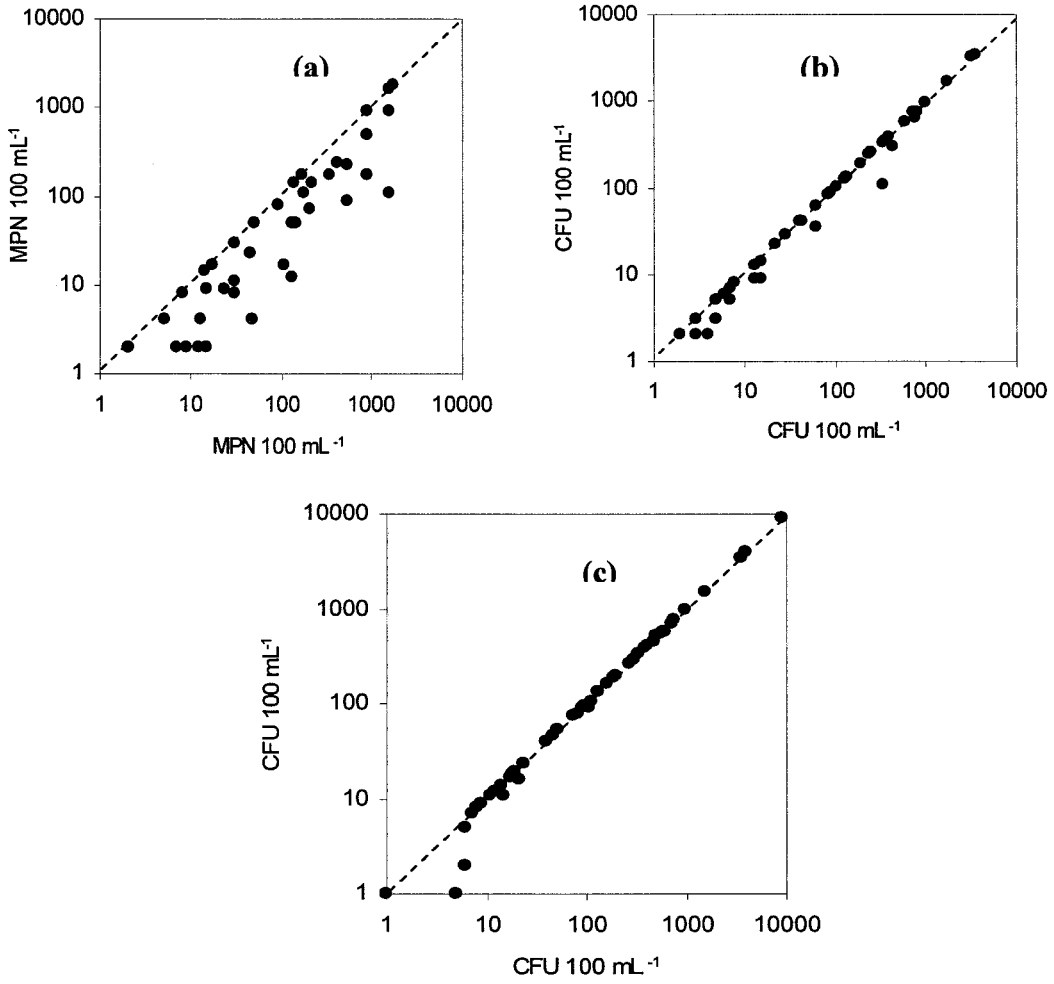


Fig. 5.14 Comparisons of presumptive and confirmed values using MPN and MF techniques for the enumeration of *Enterococcus spp.* in water samples with each data point represented for a single water sample analysed using MPN or MF method, i.e. (a) presumptive count for azide dextrose broth (X-axis) and confirmed count for azide dextrose broth (Y-axis) using MPN method, (b) presumptive count for Slanetz and Bartley agar (X-axis) and confirmed count for Slanetz and Bartley agar (Y-axis) under aerobic conditions using MF method, (c) presumptive count for Slanetz and Bartley agar (X-axis) and confirmed count for Slanetz and Bartley agar (Y-axis) under ROS-neutralised conditions using MF method. The dotted diagonal line passing through equivalent positions on the XY-axis is the line of equivalence. Note that the X-axis and Y-axis are log-transformed.

5.4 Discussion

Several different approaches have been used to aid the enumeration of injured bacteria in earlier studies. Enhancement in colony counts of sub-lethally heat-injured *Escherichia coli* O157:H7 and *Salmonella typhimurium* has been noted when a non-selective agar was overlaid for 3 h in the upper compartment of two compartment Lutriplate followed by a selective agar underlaid on the lower part of the Lutriplate (Kang and Siragusa, 1999; Kang 2002). Pre-enrichment on a non-selective medium has been used for the enumeration prior to use of a selective medium for increasing the colony count of heat-injured bacteria (Rose *et al.*, 1975; Han *et al.*, 2002). Baird-Parker (1962) introduced a medium (Baird-Parker agar) for the improved enumeration of *Staphylococcus aureus* from food, with the addition of sodium pyruvate and egg yolk emulsion to the medium to enhance the resuscitation of damaged cells (Baird-Parker, 1963; Schoeller and Ingham, 2003; Sandel and McKillip, 2004). In other studies a modified selective medium termed m-T7 agar was designed by LeChevallier *et al.* (1983) for the improved detection and resuscitation of injured total coliform bacteria using membrane filtration of drinking water samples. This medium was especially formulated to address the inhibitory nature of selective media containing bile salts or sodium deoxycholate. Introduction of this medium highlighted the shortcomings of mEndo agar commonly used for enumeration, with a lower recovery and detection rate of injured coliforms and poor differentiation between coliforms and non-coliforms (McFeters *et al.*, 1986). Furthermore, selective ingredients such as sodium deoxycholate, pararosaniline, and sodium lauryl sulphate present in m-Endo have been shown to inhibit the growth of injured coliforms (Sciff *et al.*, 1970; McFeters *et al.*, 1982; Singh *et al.*, 1990; Chilvers, 2001). The m-T7 agar recovered more faecal coliforms when compared to mFC agar with and without a lactose agar overlay (LeChevallier *et al.*, 1983;

LeChevallier and McFeters, 1985). More recently m-T7 agar medium has been used to understand the kinetics of injury of *Escherichia coli* experimentally injected in a drinking water distribution system, by calculating the difference in CFU obtained on m-T7 agar and mFC-R agar, respectively, divided by the number of CFU counted on mT7 medium to give the percentage injury (Fass *et al.*, 1996). The poor capabilities of mFC agar and m-Endo agar to recover chlorine-injured coliforms are attributed to the presence of surface-active ingredients and have been noted by Calabrese and Bissonnette (1990a), compared to m-T7 agar with and without the addition of peroxide-degrading compounds such as catalase and pyruvate. Additionally a higher recovery rate was noted for heat-injured *Staphylococcus aureus* by Ugborogho and Ingham (1994) by incubating the enrichment medium under strict anaerobic conditions, compared to aerobic conditions.

In the present study m-T7 agar was used for the resuscitation of total coliforms present in environmental water samples and its performance was compared with various selective media under aerobic and ROS-neutralised conditions. This resuscitative medium recovered more coliforms in 60% to 80% of samples compared to MacConkey agar under both sets of growth conditions (Fig. 5.1a, Fig. 5.2b; Table 5.1), while all of the other selective media i.e. mFC-R agar, m-Endo agar and mLSA agar gave consistently lower counts after screening 50 water samples for total coliforms (Fig. 5.1b-d, Fig. 5.2b-d,). The increase in coliform counts on a resuscitative medium has been noted by several researchers (LeChevallier *et al.* 1983; Fass *et al.*, 1996) and they commented that the true bacterial load could be underestimated in analysed samples by the use of conventional selective media. Enumeration under ROS-neutralised conditions, i.e. addition of pyruvate to m-T7

agar with anaerobic incubation proved to further increase the effectiveness of this medium (Fig. 5.3).

Lin (1974) suggested a pre-enrichment step using the membrane filtration assay for the enumeration of enterococci isolated from chlorinated sewage effluents. Furthermore, he compared the performance of various growth media such as brain-heart infusion broth, azide dextrose broth, peptone yeast extract casitone broth and bile broth medium (oxbile + brain-heart infusion broth) as a preliminary step for satisfactory resuscitation before placement of membrane filters on selective Slanetz and Bartley medium (Clesceri *et al.*, 1998). Maximum resuscitation was attained using 2-4 h pre-enrichment of bacteria in bile broth medium before transfer to agar medium for 46 h. Since then various researchers have suggested resuscitation techniques for the enumeration of heat-injured enterococci by incubation for 2 h on non-selective agar such as tryptic soy agar at 37°C followed by incubation for 46 h at 37°C using Slanetz and Bartley agar as an overlay medium (Steen and Eie, 1992; Domig *et al.*, 2003). Previous studies on vancomycin-resistant-enterococcus (VRE) strains from patient swabs showed better results when the swabs were placed in pre-enrichment broth when compared to direct inoculation onto selective agar (Hacek *et al.*, 2000). Recent studies using a fluorogenic probe technique for detecting *E. coli*/total coliforms and *Enterococcus* spp. from natural waters have achieved promising results after incorporation of a pre-enrichment step using peptone broth (Frahm and Obst, 2003). In the present study the inclusion of a 3 h pre-enrichment step for isolation of enterococci from water samples using bile broth medium consisting of oxbile and brain heart infusion broth recovered more bacteria under aerobic conditions in comparison to Slanetz and Bartley agar (Fig. 5.4a) and KF streptococcus agar (Fig. 5.5a) without a pre-enrichment step. Furthermore using

ROS-neutralised medium with or without a pre-enrichment step did not substantially affect the enterococci count on KF streptococcus agar (Fig. 5.5b; Table 5.2) or Slanetz and Bartley agar (Fig. 5.4b) confirming the results obtained in the Chapters 3 and 4 that ROS-neutralised conditions are effective for the enumeration of stressed bacteria. The differences between these two media with the bile broth pre-enrichment step for enterococcal enumeration were minimal under aerobic (Fig. 5.6a) and ROS-neutralised conditions (Fig. 5.6b), thus recovering an equivalent number of bacteria.

It is well established that enhanced enumeration of injured bacteria may be obtained after incorporation of peroxide-degrading compounds such as catalase and pyruvate into the growth medium (Czechowicz *et al.*, 1996; Mizunoe *et al.*, 1999; Stephens *et al.*, 2000; Khaengraeng and Reed, 2005) and this has been discussed in detail in Chapters 3 and 4. The exogenous addition of catalase or pyruvate into selective culture media used for resuscitation of injured bacteria from food and water samples have proved to increase the enumeration of physically stressed or chemically stressed bacteria, for example Baird-Parker agar used for the enumeration of *Staphylococcus aureus* contains sodium pyruvate (Schoeller and Ingham, 2003; Sandel and McKillip, 2004) and in the Chromocult coliform agar (Manafi, 2000).

The results shown in Fig. 5.4b, 5.5a-b has suggested that when ROS-neutralised medium is used then a pre-enrichment step could be omitted as the ROS-neutralised medium is sufficient for the enumeration of injured bacteria. It was noted in the present study that resuscitative medium e.g. m-T7 agar gave higher counts in over 90% of samples under ROS-neutralised conditions compared to the corresponding counts obtained under aerobic conditions (Fig. 5.3). Whereas, the differences between m-T7 agar and MacConkey agar under ROS-neutralised conditions (Fig.

5.2) were minimal, which suggests that ROS-neutralised media might solve some of the problems encountered during enumeration of injured bacteria. The use of non-selective growth media such as tryptic soy agar, nutrient agar or brain-heart infusion agar is restricted. Such growth media cannot be used for assessing and screening total coliforms and enterococci in environmental samples because of the presence of other bacterial groups in the samples (Fung *et al.*, 1995).

Researchers have noted that Slanetz and Bartley agar and KF streptococcus agar combined with the membrane filtration technique provides superior performance when compared other growth media such as pfizer selective enterococcus agar, kanamycin aesculin azide agar, bile aesculin agar, thallous acetate agar and mitis-salivarius agar (e.g. Dionisio and Borrego, 1995; Domig *et al.*, 2003). In the present study a selective medium which could recover high numbers of sub-lethally injured bacteria was identified; MacConkey agar was used for the enumeration of *Escherichia coli* while Slanetz and Bartley agar was used for the enumeration of *Enterococcus* spp. from water samples. These growth media using the MF format were incubated under aerobic and ROS-neutralised conditions and their potential to recover bacteria was compared with growth media commonly used in the MPN method. It was noted that presumptive MPN values for MacConkey broth gave higher results in 63% of water samples compared to the results obtained for samples aerobically incubated MacConkey agar in the MF assay (Fig. 5.7a). However, many samples of these presumptive positives for MPN were confirmed as false-positives for *E. coli* using HiChrome *E. coli* agar. Statistical analysis using the Wilcoxon signed rank test showed a significant difference between MPN and MF-aerobic assays with the MF-aerobic assay giving statistically significant higher confirmed values (Fig. 5.7 b).

Similar results were obtained for *Enterococcus* spp. (Fig. 5.11a-b) with significantly higher values obtained for MF (Slanetz and Bartley agar) incubated aerobically (Fig. 5.11b) after confirmation on aesculin agar compared with MPN (azide dextrose broth). Comparisons of MPN (azide dextrose broth) with MF (Slanetz and Bartley agar) ROS-neutralised medium for presumptive (Fig. 5.8a) and confirmed values (Fig. 5.8b) showed the latter to be more effective for the enumeration and isolation of *Enterococcus* spp. from water samples. The ROS-neutralised medium showed significant higher values for presumptive and confirmed data compared to the corresponding values obtained by using aerobic medium (Fig. 5.13a-b) or MPN-azide dextrose broth (Fig. 5.12a-b).

The MF method is considered as a method of choice in standard methods in US as it gives results on a quantitative basis (Clesceri *et al.*, 1998; Rompre *et al.*, 2002). The results of the present study indicate that the efficiency of the MF method could be further improved by using growth medium incubated under ROS-neutralised conditions which was effective in enhancing the count for faecal indicator bacteria in water compared with the MPN assay or with conditions for the MF assay (Baylis *et al.*, 2000; Spiegeleer *et al.*, 2004; Khaengraeng and Reed, 2005).

Chapter 6

Development of a novel broth-based medium for the enumeration of injured *E. coli*

6.1 Introduction

The two main culture-based approaches currently used in the US (Clesceri *et al.*, 1998) and the UK (Anon., 2002) for the detection and enumeration of faecal indicator bacteria are, firstly, multiple tube fermentation or most probable number (MPN) and, secondly, membrane filtration (MF). These approaches have been described in detail in Chapter 1 and are based on properties such as acid or gas production from the fermentation of lactose at the initial presumptive stage followed by confirmatory and completed stages requiring up to three days. Such tests are relatively easy to perform and cost-effective (WHO, 2004b), but they are also tedious and time-consuming (Edberg *et al.*, 1990; Argent *et al.*, 1991; Rice *et al.*, 1991; Chilvers, 2001). Methods based on differential selective media such as the m-Endo medium recommended by the US and the Indian authorities (Clesceri *et al.*, 1998; Vasudeva and Pathak, 1998), or mLSA medium recommended in the UK (Anon., 2002), have a number of drawbacks for total coliforms and/or *Escherichia coli* such as lack of specificity, poor detection rate, interference by antagonistic organisms and low recovery rate, concerning especially for sub-lethally injured bacteria (McFeters *et al.*, 1982; Burlingame *et al.*, 1984; Sartory and Howard, 1992; Sartory, 1995; Chilvers *et al.*, 2001; Rompre *et al.*, 2002). Furthermore, new culture-based approaches such as defined enzyme substrate technology involving the use of fluorogenic and chromogenic enzyme substrates in non-selective and selective media have been introduced in both MPN and MF assays (Edberg and Edberg, 1988; Brenner *et al.*, 1993; Eckner, 1998; Manafi, 2000), making it possible to detect and identify target micro-organisms in primary isolation media, thus thereby reducing the time period normally required for confirmatory and completed tests (Chapter 1).

The most recent revision to UK methods (Anon., 2002) suggests a more accurate and sensitive miniaturised MPN method utilising multiwell assay trays that can provide more quantitative results since it utilises a large number of test wells, such as a 96-well microplate or multititre tray for examination of a single sample. The 96-well microplate assay was initially introduced by Maul and Block (1983) and for miniaturized fluorogenic assays by Hernandez *et al.* (1991a) proving to be a improvement in MPN assay by giving an approximately normalised MPN value, increasing the number of replicates and dilutions and utilising small volumes of 100-200 microlitres of sample (Hernandez *et al.*, 1991b; Hernandez *et al.*, 1993; Chilvers, 2001). Furthermore, the MPN multiwell calculations can be performed using software such as the MPN calculator, which also gives 95% confidence limits that provide a reliable estimate of bacterial number (Garthright and Blodgett, 2003; Curiale, 2004). Commercial products such as the Colilert QuantiTray system (IDEXX) are based on the 96-well tray format using powdered basal medium containing the chromogen *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) and the fluorogen 4-methylumbelliferyl-β-glucuronide (MU-GLUC) for the identification of bacteria such as total coliforms and *E. coli* giving a positive reaction because of the presence of enzymes β-galactosidase and β-glucuronidase respectively (Edberg *et al.*, 1990; Manafi, 2000).

Researchers have outlined and studied the inhibitory nature of ingredients commonly incorporated for selective and differential purposes in culture media such as bile salts in MacConkey agar (Anon., 2002), sodium deoxycholate in mEndo agar, or sodium lauryl sulfate in membrane lauryl sulphate broth (McFeters *et al.*, 1982; Babich and Babich, 1997). Such components are known to inhibit growth of debilitated bacterial cells (Calabrese and Bissonette, 1990a). Attempts have been made in different

studies to introduce modifications in the selective media used for enumeration such as the development of resuscitative medium m-T7 agar (Chapter 5) or the use of a non-selective pre-enrichment step or by incorporation of components normally beneficial to the target micro-organism (McFeters *et al.*, 1986; Han *et al.*, 2002) to increase the sensitivity of detection. Sodium heptadecyl sulphate or Tergitol 7 a selective ingredient of the membrane filtration medium, m-T7 agar is a surfactant that inhibits the growth of many Gram-positive and Gram-negative bacteria, except coliforms (Scherer, 1966; Hinds and Howard, 1974; Freier and Hartman, 1987; Ciebin *et al.*, 1995). It was originally introduced in 1946 and termed tergitol agar that was later modified by the addition of triphenyltetrazolium chloride (TTC) to aid in the detection of *Escherichia coli* (LeChevallier *et al.*, 1983). This medium has the characteristics of producing consistent colony morphology for coliform bacteria such as *E. coli* (LeChevallier *et al.*, 1983; 1985) and thus has several advantages in the selection and differentiation of coliforms from non-coliforms compared to mEndo agar (Hinds and Howard, 1974). Tergitol-7 individually added to selective media is known to enrich the growth and isolation rate of some coliforms e.g. by increasing the number of micro-organisms such as *Salmonella* from pork sausages (Morris and Dunn, 1970; Fluit *et al.*, 1993). It has been incorporated into conventional media for improved recovery and differentiation of faecal coliforms from environmental samples; such media include tergitol agar, m-T7 agar (LeChevallier and McFeters, 1985), peptone tergitol glucuronide agar (Freier and Hartman, 1987), lactose tergitol 7 TTC agar (Niemi *et al.*, 2001), Chromocult coliform agar (Manafi, 2000) and PTX agar (Mihoub *et al.*, 2003). A constituent such as Tergitol 7 has the potential to be less inhibitory to injured bacteria than selective agents in traditional media, such as bile or inhibitory dyes (Clesceri *et al.*, 1998). This chapter describes the experiments carried out to develop a broth-based medium for the detection and enumeration of

sub-lethally injured *E. coli,* based on properties such as non-inhibitory selective agents along with, appropriate resuscitative and diagnostic constituents.

The specific objectives of the present study were:

1. To develop a novel broth-based medium for the detection and enumeration of *E. coli.* The laboratory experiments were carried out to find out the most effective ingredients that would help in enumerating injured micro-organisms. The formulation of the novel medium was based on choice of an appropriate selective ingredient such as Tergitol 7, bile salts or sodium lauryl sulphate, a differential agent based on defined enzyme substrate technology and a resuscitative agent such as sodium pyruvate, DTT or sodium thioglycollate to act as a ROS scavenger.
2. To use this novel broth-based medium in an MPN multiwell format and compare it with conventional plating method for the enumeration of injured *E. coli* under different sets of growth conditions (aerobic/anaerobic) and by using various selective media.

6. 2 Materials and Methods

Pure cultures of the strain *Escherichia coli* NCTC8912 were used throughout the experiments conducted at Northumbria University (Chapter 2). A bacterial cell suspension (Chapter 2) was diluted to 1: 1000, i.e. 300 µL of rinsed cells was added to 300 mL of sterile distilled water in a transparent plastic bottle of volume 500 mL and incubated in a UV-A cabinet (Chapter 4) to determine the time required to give sub-lethal injury. Timed samples were taken from control (unexposed) and UV-A exposed samples and processed by performing serial dilutions using the MPN multiwell assay along with a standard plate count. In both cases, serial decimal dilutions down to 10^{-5} were performed as described in detail in Chapter 2. Comparisons were made between assays after conversion of the values (MPN per mL or plate count CFU per mL) obtained by each method into a mean value per mL. Experiments were conducted using different selective and diagnostic components incorporated in basal non-selective nutrient medium for formulation and development of field medium that should constitute properties such as being selective, diagnostic and resuscitative as described below:

6.2.1 Evaluation of various selective agents

Individual selective ingredients most commonly incorporated into enumeration media for isolation of *E. coli* were added to nutrient broth for MPN multiwell assay and to nutrient agar for plate counts. These ingredients were used according to the amount present in commercial selective media, such as sodium lauryl sulphate 0.02 g L^{-1}, as in membrane lauryl sulphate medium (Anon., 2002), bile salts 5 g L^{-1} (Merck VWR, Darmstadt, Germany) as in MacConkey agar (McFeters *et al.*, 1982), Tergitol-7 0.1 g L^{-1} (Sigma, Dorset, UK) as in m-T7 agar and Chromocult agar (LeChevallier and McFeters, 1985; Clesceri *et al.*, 1998).

A stock solution of Tergitol-7 was initially prepared by adding 1g to 100 mL of autoclaved sterile water in a volumetric flask. Broth media for multiwell MPN were prepared prior to autoclaving at two and half times normal strength, added to 100 mL distilled water in the amounts described below:

- Nutrient broth 6.25 g
- Nutrient broth 6.25 g + 0.05% w/v sodium pyruvate 0.125 g
- Nutrient broth 6.25 g + sodium lauryl sulphate 0.05 g
- Nutrient broth 6.25 g + sodium lauryl sulphate 0.05 g + 0.05% w/v sodium pyruvate 0.125 g
- Nutrient broth 6.25 g + bile salts 1.25g
- Nutrient broth 6.25 g + bile salts 1.25g + 0.05% w/v sodium pyruvate 0.125 g
- Nutrient broth 6.25 g + Tergitol-7 0.025 g
- Nutrient broth 6.25 g + Tergitol-7 0.25 g + 0.05% w/v sodium pyruvate 0.125 g

Similarly for the plate count method single-strength nutrient agar and individual ingredients were added to 400 mL of sterile distilled water in the amounts described below:

- Nutrient agar 11.2 g
- Nutrient agar 11.2 g + 0.05% w/v sodium pyruvate 0.2 g
- Nutrient agar 11.2 g + sodium lauryl sulphate 0.08 g
- Nutrient agar 11.2 g + sodium lauryl sulphate 0.08 g + 0.05% w/v sodium pyruvate 0.2 g
- Nutrient agar 11.2 g + bile salts 2g

- Nutrient agar 11.2 g + bile salts 2g + 0.05% w/v sodium pyruvate 0.2 g
- Nutrient agar 11.2 g + Tergitol-7 0.04 g
- Nutrient agar 11.2 g + Tergitol-7 0.04 g + 0.05% w/v sodium pyruvate 0.2 g

Following inoculation, all multiwell trays and plates were incubated under standard aerobic conditions for 24 h and in an anaerobic jar for 48 in a conventional incubator at 37°C.

6.2.2 Effects of peroxide-neutralisation / ROS-neutralisation

After selection of the least inhibitory selective agent, i.e. Tergitol-7 which recovered the highest number of UV-A exposed *E. coli* in the initial experiments, the next step was to compare different counts of the UV-A injured cells in the MPN multiwell assay and plate count assay using nutrient medium with Tergitol-7 firstly supplemented with and without 0.05 % w/v sodium pyruvate, secondly by using DTT (10 mmol L^{-1} = 1.542 g L^{-1}, Chapter 3) that lowers the redox potential of the medium with and without 0.05 % w/v sodium pyruvate or thirdly by including sodium thioglycollate (2 g L) which is used as reducing agent in the growth medium with and without 0.05 % w/v sodium pyruvate supplementation. The entire growth medium (multiwell trays and Petri plates) were incubated under standard aerobic conditions and under anaerobic conditions using an anaerobic jar or using pre-reduced medium in an anaerobic cabinet (Chapter 3), to establish the effect of ROS-neutralisation.

6.2.3 Comparison of novel broth-based medium with standard media after selection of an appropriate diagnostic reagent

Initially nutrient broth was used for development of the field medium with and without addition of selective agent Tergitol-7 and resuscitative agent, i.e. 0.05% w/v sodium pyruvate. But for final development a basal medium and diagnostic agent had to be added to make the medium both differential and specific. The selection of the diagnostic ingredient was decided to provide specific detection of *E. coli*. This ingredient was based on defined enzyme substrate technology for β-glucuronidase as discussed in detail in Chapter 1, namely 5-bromo-4 chloro-3-indolyl-β-glucuronide (X-GLUC). In preliminary experiments the optimum concentration of X-GLUC giving a clear-cut green colour was chosen after incorporating in the basal medium at concentrations ranging from 50 mg L^{-1} to 150 mg L^{-1}. Thus the initial composition of the novel broth-based medium for preliminary laboratory experiments was as follows for single-strength medium

(in g L^{-1}) :

(NTX) 25 g of Nutrient broth, 0.1 g of Tergitol 7, 5 g of lactose, 100 mg of 5-bromo-4 chloro-3-indolyl-β-glucuronide (X-GLUC).

The composition of various selective media namely MacConkey broth (described in Chapter 2), minerals modified glutamate medium (mG), lauryl tryptose broth (LTB) and EC (EC) broth for single-strength medium in g L^{-1} are described below (in g L^{-1}):

- minerals modified glutamate medium constituted as 20.0 g of lactose, 12.7 g of sodium glutamate, 40 mg of arginine monohydrochloride, 48 mg of aspartic acid, 40 mg of cystine, 500 mg of sodium formate, 1.8 g of di-

potassium hydrogen phosphate, 5 g of ammonium chloride, 200 mg of magnesium sulphate heptahydrate, 20 mg of calcium chloride dihydrate, 20 mg of iron (III) citrate, 2 mg of thiamine (aneurin hydrochloride), 2 mg of nicotinic acid, 2 mg of pantothenic acid and 2 mL of bromocresol purple (1 % m/v ethanolic solution).

- Lauryl tryptose broth constituted as 20 g of tryptose, 5 g of sodium chloride, 2.75 g of di-potassium hydrogen phosphate, 2.75 g of potassium di-hydrogen phosphate, 0.1 g sodium lauryl sulfate, 5 g of lactose and 0.05 g of phenol red.

- EC broth contained 20 g of peptone from casein, 5 g of lactose, 1.5 g of bile salt mixture, 5 g of sodium chloride, 4 g of di-potassium hydrogen phosphate and 1.5 g of potassium dihydrogen phosphate.

The various selective media were compared with NTX medium in MPN multiwell trays incubated under aerobic conditions with and without 0.05% sodium pyruvate. Duplicate MPN multiwell trays were used for each medium. The results were calculated per mL using the MPN calculator software (Curiale, 2004) as described in Chapter 2.

6.3 Results

6.3.1 Evaluation of various selective agents

Figure 6.1 shows the results obtained for *E. coli* NCTC8912 prior to UV-A exposure at 0 h, with enumeration onto aerobic unsupplemented medium, or peroxide-neutralised medium, or unsupplemented medium in an anaerobic jar or ROS-neutralised medium (pyruvate-supplemented medium in an anaerobic jar) with and without various selective agents namely Tergitol-7, bile salt and sodium lauryl sulphate incorporated in nutrient agar in plate count method (Fig. 6.1a) and nutrient broth in multiwell MPN method (Fig. 6.1b). The initial values showed no differences between the non-selective medium (nutrient agar or nutrient broth) with and without various selective agents (Tergitol-7, bile salt and sodium lauryl sulphate) under all sets of enumeration conditions, in agreement with the results obtained in Chapter 3 (Fig. 3.8) for initial plate counts using non-selective and selective media. Thus the results using broth-based multiwell assay also show that prior to any sub-lethal injury, the cells were healthy and therefore gave similar value to those of nutrient agar.

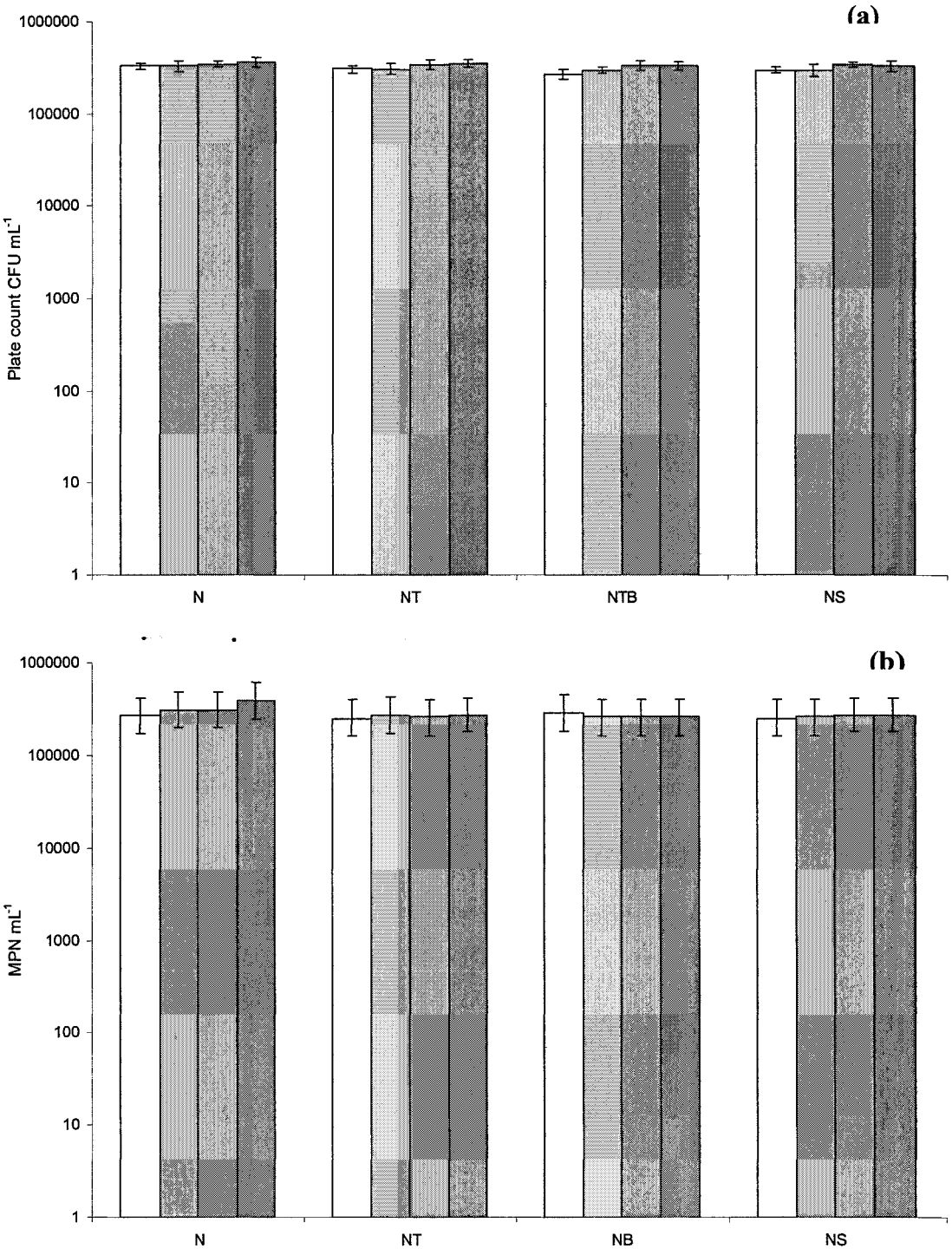


Fig. 6.1 Evaluation of various selective agents prior to UV-A exposure (0 h) (a) agar-based media, (b) broth-based media using nutrient agar or nutrient broth with and without selective agents such as Tergitol-7 (NT), bile salts (NB), sodium lauryl sulphate (NS) under aerobic conditions (unshaded bars), peroxide-neutralised (pyruvate-supplemented medium) conditions (light-grey bars), anaerobic conditions (medium grey-bars) and ROS-neutralised conditions (anaerobic conditions + sodium pyruvate; dark-grey bars). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

Fig. 6.2 shows results for 2 h UV-A irradiated *E. coli* NCTC8912 cells enumerated under various growth conditions and selective agents using agar-based assay (Fig. 6.2a) and MPN multiwell broth-based assay (Fig. 6.2b). The results showed that there were substantial decrease in the values compared to the corresponding initial values (Figure 6.1a-b), with the reduction varying from around 100-fold to 25000-fold depending upon the enumeration conditions used in agar and broth-based assays. Under aerobic conditions in plate count and MPN multiwell assay, unsupplemented nutrient agar/broth, recovered the highest number of *E. coli* cells followed by Tergitol-7-supplemented medium and bile salt-supplemented medium, while sodium lauryl sulphate as in previous experiments (Chapter 4) gave lowest values. In peroxide-neutralised media an increase in number was noted for all media, with somewhat higher increases noted for MPN multiwell assays compared to plate count assays. Anaerobic media when compared with aerobic medium showed a lesser increase than for pyruvate-supplemented medium. In plate count method ROS-neutralised conditions displayed the highest recovery rate with the trend of values obtained with and without added components to be nutrient agar > nutrient agar-Tergitol-7 > nutrient agar-bile salt > nutrient agar- sodium lauryl sulphate. In the MPN multiwellmethod ROS-neutralised medium in case of nutrient broth with or without Tergitol-7 and bile salts giving similar high values with sodium lauryl sulphate giving somewhat lower counts. Thus for the broth-based medium (MPN values) minimal differences were seen between (i) peroxide-neutralised, (ii) anaerobic incubated, (iii) ROS-neutralised medium. The differences were also smaller compared to aerobic broth medium, in contrast to aerobic agar-based medium where the differences between the various conditions were larger.

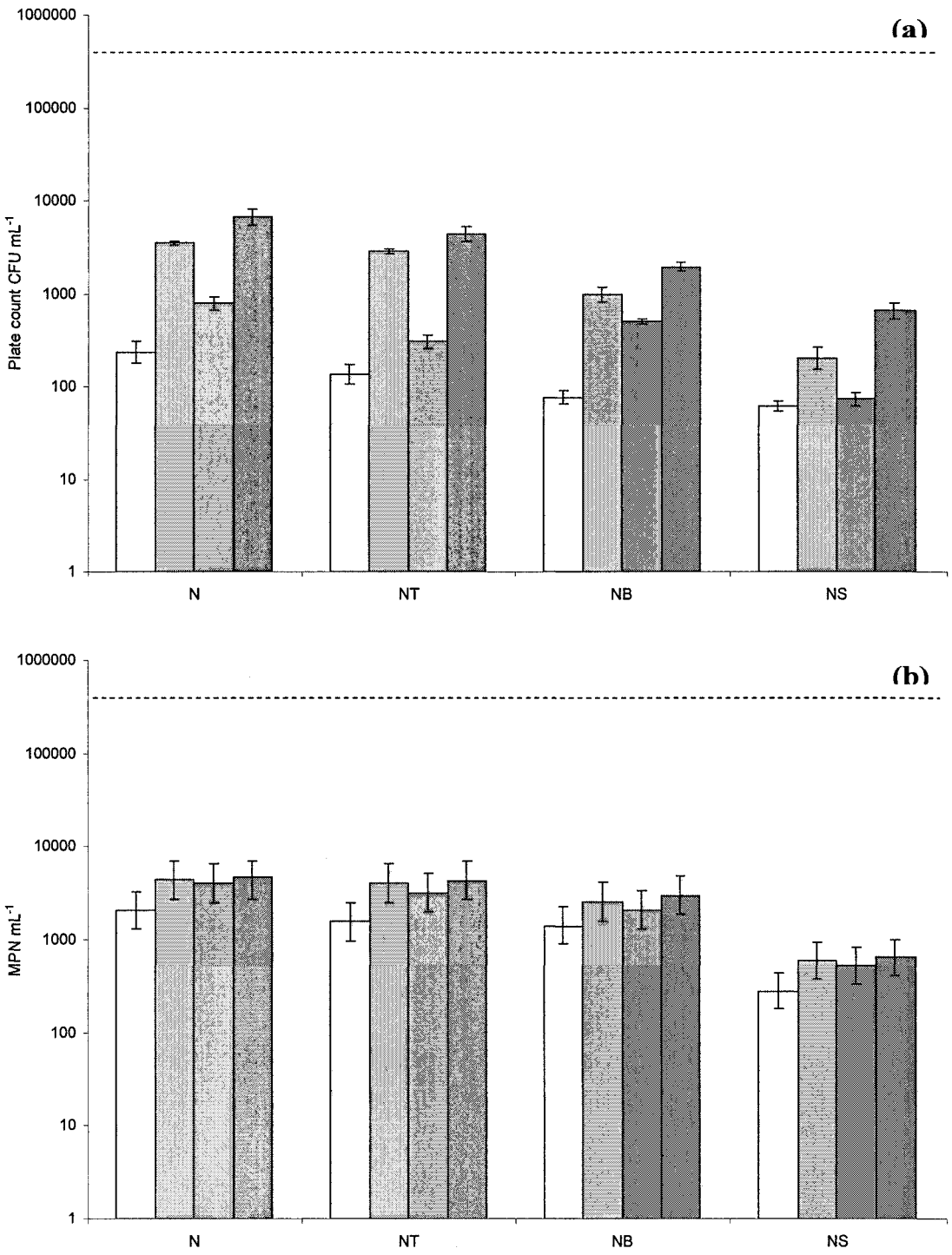


Fig. 6.2 Evaluation of various selective agents after 2 h UV-A exposure
(a) agar-based media, (b) broth-based media using nutrient agar or nutrient broth with and without selective agents such as Tergitol-7 (NT), bile salts (NB), sodium lauryl sulphate (NS) under aerobic conditions (unshaded bars), peroxide-neutralised (pyruvate-supplemented medium) conditions (light-grey bars), anaerobic conditions (medium grey-bars) and ROS-neutralised conditions (anaerobic conditions + sodium pyruvate; dark-grey bars). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

Figure 6.3 shows results for 3 h UV-A irradiated *E. coli* NCTC8912 cells enumerated using similar growth media and growth conditions as described in Fig. 6.1 and 6.2. The results showed reduction in values compared to the initial inoculum. The overall broad trends in results was similar to that obtained in Fig. 6.2 in case of plate count method (Fig. 6.3a) or MPN multiwell method (Fig. 6.3b) with the aerobic unsupplemented medium giving the lowest value, followed by unsupplemented medium incubated in an anaerobic jar, then peroxide-neutralised medium and ROS-neutralised medium giving maximum growth. In case of various selective agents only Tergitol-7 reached the values similar to that obtained for non-selective nutrient agar/broth media under all four sets of growth conditions, bile salt supplemented nutrient agar/broth media gave intermediate values, while sodium lauryl sulphate gave the lowest values. The plate count method using nutrient agar with and without Tergitol-7 as a selective agent gave results equivalent to broth-based method only in the case of peroxide-neutralised and ROS-neutralised conditions. When bile salts or sodium lauryl sulphate were used as a selective agent, the plate count method gave results equivalent to broth-based method only under ROS-neutralised conditions.

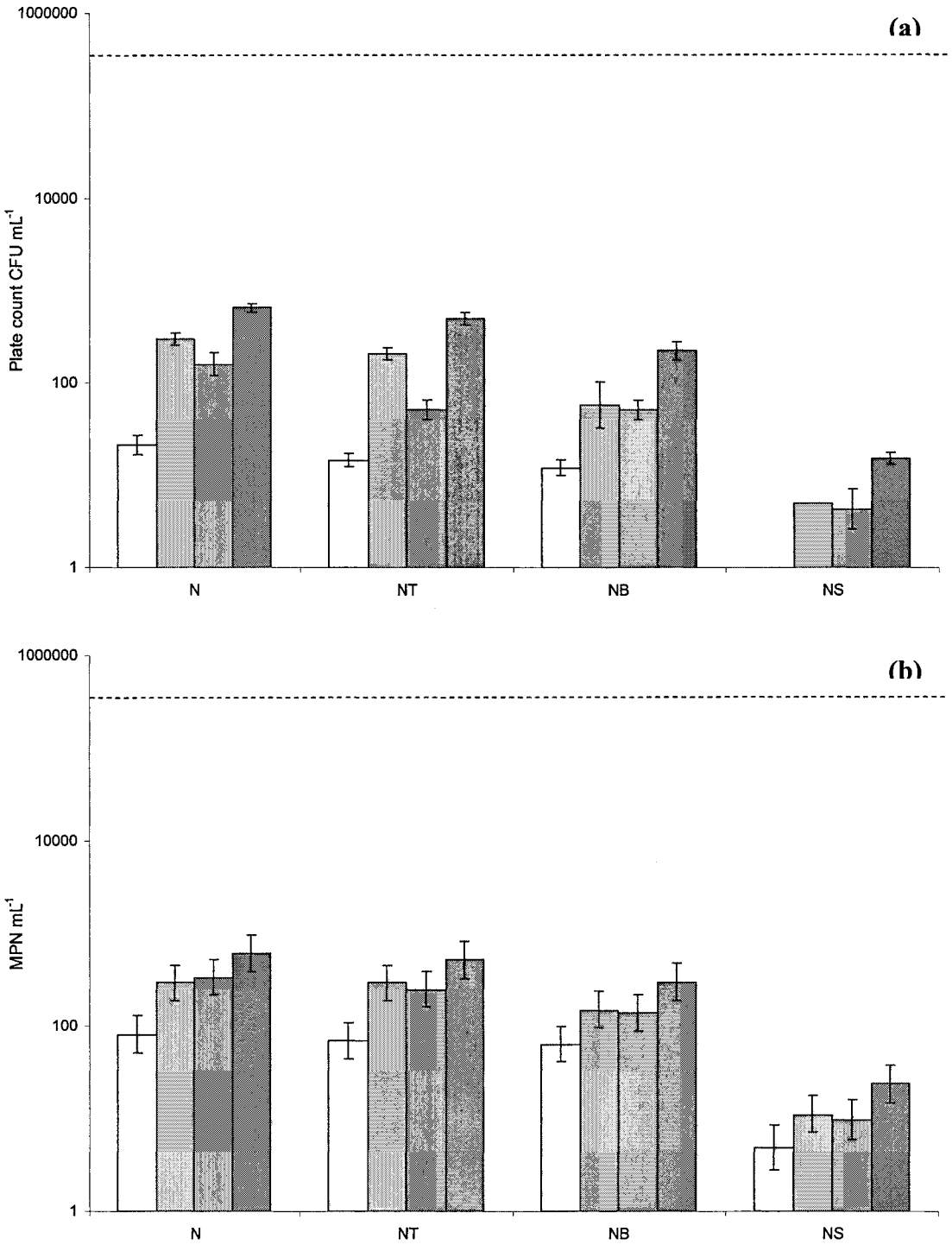


Fig. 6.3 Evaluation of various selective agents after 3 h UV-A exposure
(a) agar-based media, (b) broth-based media using nutrient agar or nutrient broth with and without selective agents such as Tergitol-7 (NT), bile salts (NB), sodium lauryl sulphate (NS) under aerobic conditions (unshaded bars), peroxide-neutralised (pyruvate-supplemented medium) conditions (light-grey bars), anaerobic conditions (medium grey-bars) and ROS-neutralised conditions (anaerobic conditions + sodium pyruvate; dark-grey bars). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

6.3.2 Effects of peroxide-neutralisation/ ROS-neutralisation

Figure 6.4 shows results for 2h UV-A irradiated *E. coli* NCTC8912 enumerated under three sets of conditions namely aerobic, anaerobic (anaerobic jar) and by using pre-reduced medium kept in anaerobic cabinet for 24 h prior to use, with and without 0.05% w/v sodium pyruvate and Tergitol-7 in plate count nutrient agar format (Fig. 6.4a) and MPN multiwell-based nutrient broth (Fig. 6.4b). Addition of Tergitol 7 to nutrient broth in broth-based multiwell assay did not have any inhibitory effect under aerobic and anaerobic conditions in an anaerobic jar and an anaerobic cabinet. Comparing plate count agar and MPN multiwell methods, higher value was obtained for nutrient broth with and without pyruvate and Tergitol-7 compared with corresponding agar plate count method under all three sets of growth conditions. Under these three sets of growth conditions MPN multiwell methods gave 100% higher values when compared with the agar plate count method.

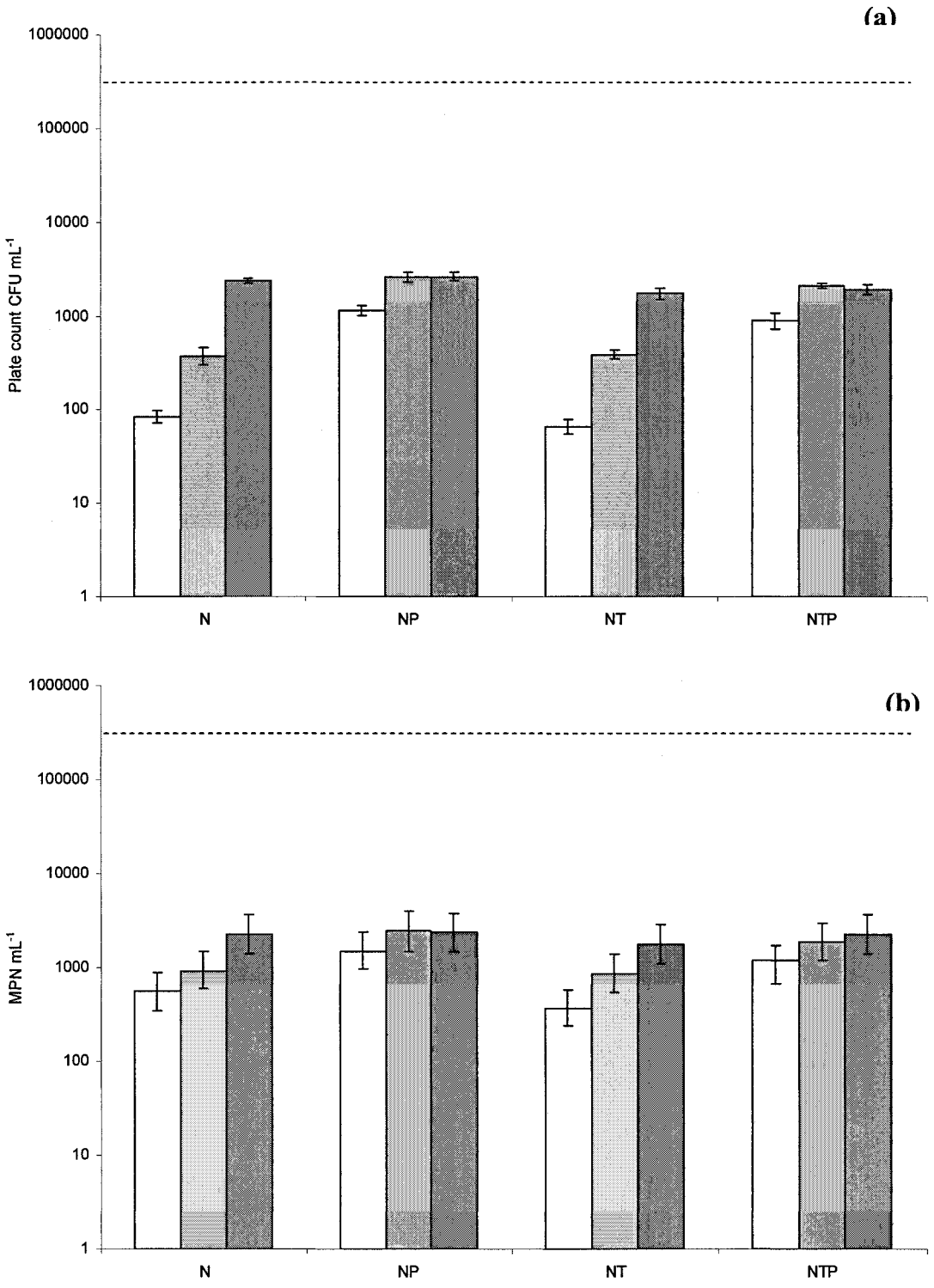


Fig. 6.4 Effects of pyruvate/ ROS-neutralisation on 2 h UV-A exposed *E. coli* NCTC8912 using Tergitol-7 as a selective agent (a) agar-based media, (b) broth-based media enumerated on nutrient agar/broth (N), and nutrient agar/broth plus 0.05% sodium pyruvate (NP), or nutrient agar/broth plus Tergitol-7 (NT), or nutrient agar/broth plus Tergitol-7 and 0.05% sodium pyruvate (NTP) incubated either aerobically (unshaded bars), or in an anaerobic jar (light grey bars), or in an anaerobic cabinet using pre-reduced medium (dark grey bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

Figure 6.5 shows results obtained for *E. coli* strain after 2 h UV-A exposure and enumerated under four sets of growth conditions, i.e. using aerobic unsupplemented medium, or peroxide-neutralised medium, or unsupplemented medium in an anaerobic jar or ROS-neutralised medium (pyruvate-supplemented medium in an anaerobic jar). The growth medium was supplemented with and without, (i) Tergitol-7 and, (ii) DTT. The agar-based medium (Fig. 6.5a) and the broth-based medium (Fig. 6.5b) with and without Tergitol-7 gave higher values after inclusion of DTT compared to the equivalent medium without DTT. In the case of the peroxide-neutralised, anaerobic and ROS-neutralised conditions, agar and broth-based media, with and without Tergitol-7 and pyruvate showed minimal increases in the value when spread plated with DTT. The aerobic unsupplemented medium with DTT in the broth-based media (multiwell assay) reached almost to the value obtained for peroxide-neutralised, anaerobic and ROS-neutralised medium (both with and without Tergitol-7), while no such increases were seen for the medium with or without DTT for peroxide-neutralised, anaerobic or ROS-neutralised conditions. Thus the results show that DTT has a positive effect only under aerobic conditions on unsupplemented media and has no substantial effect in all other growth conditions used for both methods (Chapter 3; Fig.3.3). Therefore DTT was not added to the novel medium.

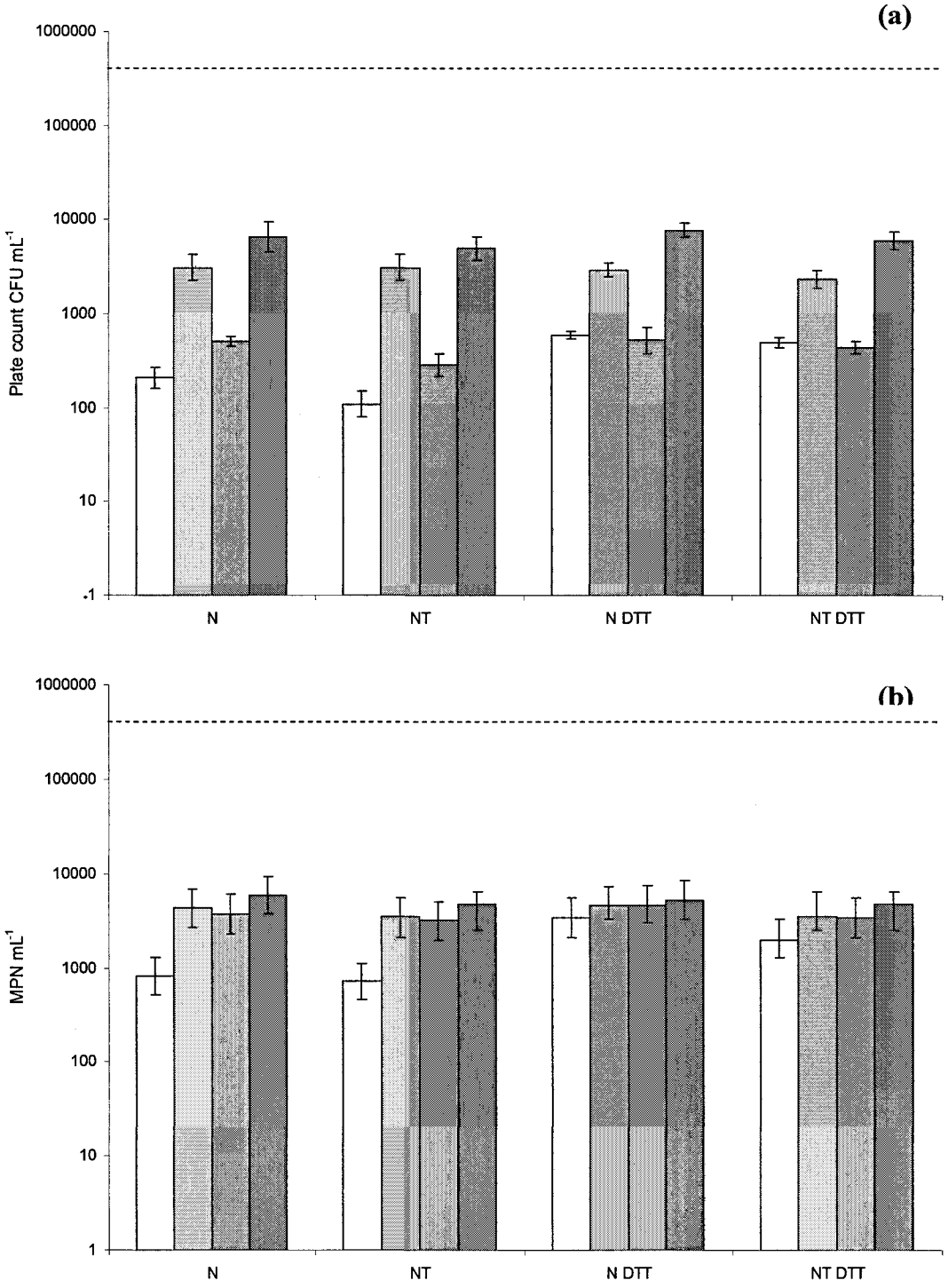


Fig. 6.5 Effects of pyruvate/ ROS-neutralisation on 2 h UV-A exposed *E. coli* NCTC8912 using Tergitol-7 and DTT (a) agar-based media, (b) broth-based media enumerated on nutrient agar/broth (N), and nutrient agar/broth plus Tergitol-7 (NT), or nutrient agar/broth plus DTT (NDTT), and nutrient agar/broth plus Tergitol-7 plus DTT (NTDTT), incubated either aerobically (unshaded bars), or peroxide-neutralised (pyruvate-supplemented medium) conditions (light-grey bars), anaerobic conditions (medium grey-bars) and ROS-neutralised conditions (anaerobic conditions + sodium pyruvate; dark-grey bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

Figure 6.6 shows results obtained for *E. coli* UV-A illuminated cells, enumerated under three sets of enumeration conditions, i.e. either aerobically, or anaerobically in an anaerobic jar, or using pre-reduced medium kept prior to use in anaerobic cabinet. The medium was supplemented with and without either, (i) 0.05% w/v sodium pyruvate, (ii) Tergitol-7 and, (iii) sodium thioglycollate. The results of aerobic-unsupplemented growth media (plate count and MPN multiwell) with or without Tergitol-7 gave a higher value after the inclusion of sodium thioglycollate. The aerobic-pyruvate supplemented growth media showed minimal differences whether sodium thioglycollate was included in media or not. The anaerobic-unsupplemented growth media showed an increase in the value in the case of the agar plate method, while the increases in the MPN value in multiwell assay were not substantial. The anaerobic-pyruvate supplemented and pre-reduced growth media (ROS-neutralised conditions) showed minimal differences in the value with and without inclusion of sodium thioglycollate, thus demonstrating that increases in value were obtained only under aerobic and anaerobic unsupplemented media. Thus sodium thioglycollate offered no benefit in pyruvate-supplemented media and was not considered further.

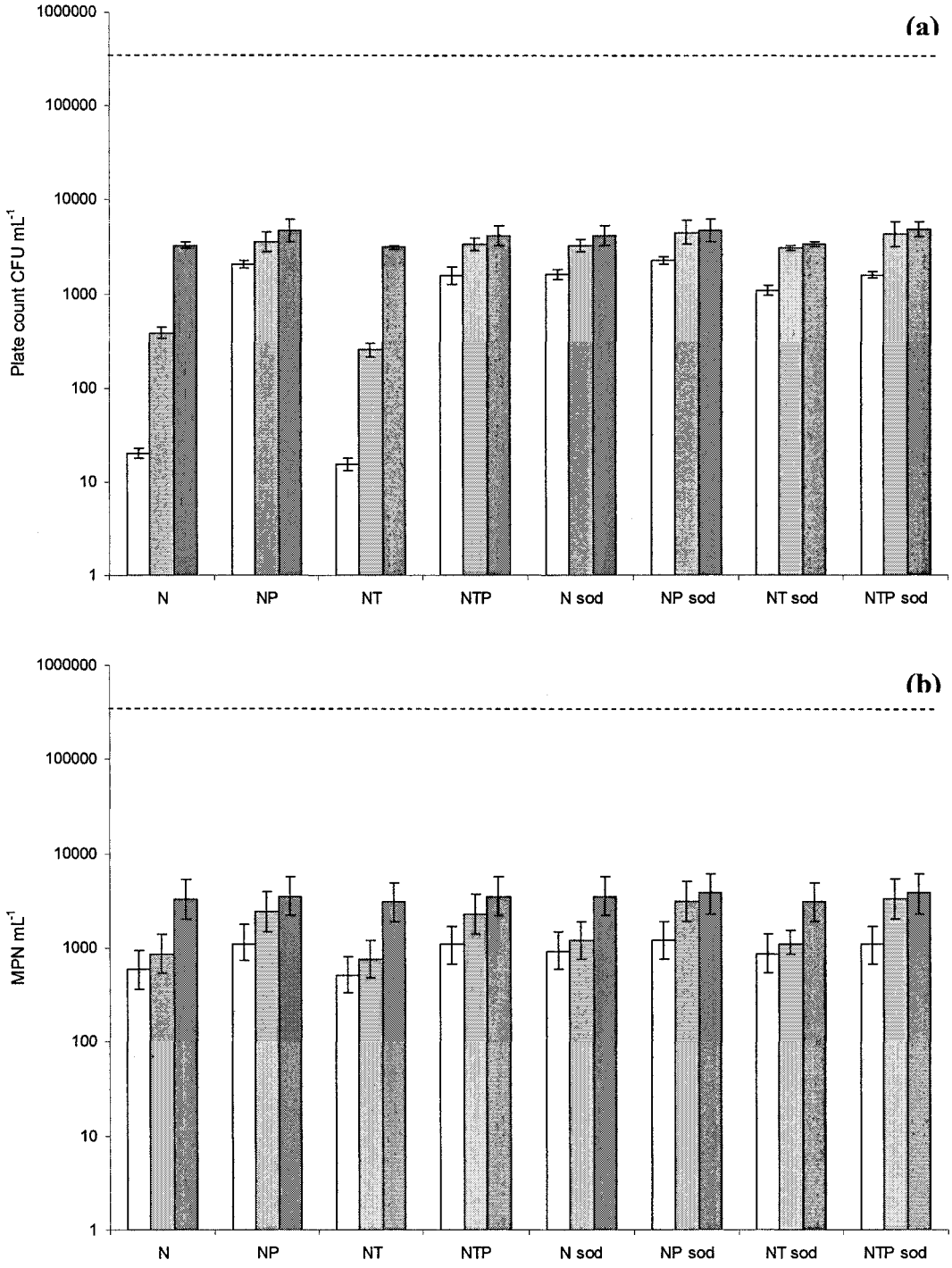


Fig. 6.6 Effects of pyruvate/ ROS-neutralisation on 2 h UV-A exposed *E. coli* NCTC8912 using Tergitol-7 and sodium thioglycollate in growth medium (a) agar-based media, (b) broth-based media, enumerated on nutrient agar/broth (N), and nutrient agar/broth plus 0.05% sodium pyruvate (NP), or nutrient agar/broth plus Tergitol-7 (NT), or nutrient agar/broth plus Tergitol-7 and 0.05% sodium pyruvate (NTP), or nutrient agar/broth plus sodium thioglycollate (N sod), or nutrient agar/broth plus sodium thioglycollate and 0.05% sodium pyruvate (NP sod), or nutrient agar/broth plus Tergitol-7 and sodium thioglycollate (NT sod), or nutrient agar/broth plus Tergitol-7, 0.05% sodium pyruvate and sodium thioglycollate (NTP sod), incubated either aerobically (unshaded bars), or in an anaerobic jar (light grey bars), or in an anaerobic cabinet using pre-reduced medium (dark grey bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

6.3.3 Comparison of novel broth-based medium with standard media after selection of an appropriate diagnostic reagent

A diagnostic reagent X-GLUC in concentration of 100 mg L^{-1} was added to the basal medium (chromocult coliform agar; Frampton *et al.*, 1988; Merck VWR, Darmstadt, Germany). The results obtained from the previous experiments carried out using the MPN multiwell broth-based assay show that the growth medium with Tergitol-7 gives results broadly comparable to the medium without it, especially in pyruvate-supplemented media. Thus the selective agent Tergitol-7 does not substantially inhibit the growth of injured bacteria.

Figure 6.7a represents results for 2 h UV-A exposed *E. coli* cells enumerated under aerobic conditions with and without 0.05% sodium pyruvate using nutrient broth with the addition of Tergitol-7 and X-GLUC, compared with various other standard selective media such as MacConkey broth, minerals modified glutamate medium broth, lauryl tryptose broth and EC broth in duplicate MPN multiwell assays. The aerobic results show that a combination of non-selective nutrient broth, Tergitol-7 and X-GLUC termed NTX medium, recovered the highest number of UV-A-exposed *E. coli* compared to all conventional selective media. For the commercial selective media MacConkey broth gave the highest MPN value followed by minerals modified glutamate medium, with lauryl tryptose and EC broth giving the lowest value. In peroxide-neutralised conditions, i.e. after supplementation of pyruvate a further increase in MPN value was noted from the corresponding medium without it. The peroxide-neutralised conditions also increased the MPN value on various selective media but none of these reached to the value obtained for pyruvate-supplemented NTX (See Plate 6.1).

Figure 6.7b shows results for the same experiment as displayed in Fig. 6.7a, but after 3 h UV-A exposure, enumerated using aerobic growth conditions with and without 0.05% sodium pyruvate using NTX and various other selective media. Overall 3 h of exposure gave a further reduction in MPN value which was below detectable levels for EC broth and lauryl tryptose broth under aerobic conditions, whereas EC broth but not lauryl tryptose broth in peroxide-neutralised conditions showed increases in MPN value. The broad trends in the pattern of results for all other media were similar to those shown in Fig. 6.7a, with NTX in peroxide-neutralised conditions giving the highest MPN value and with good agreement between duplicate MPN values. Thus these results show that pyruvate-supplemented NTX to be the best resuscitative medium of the tested here.

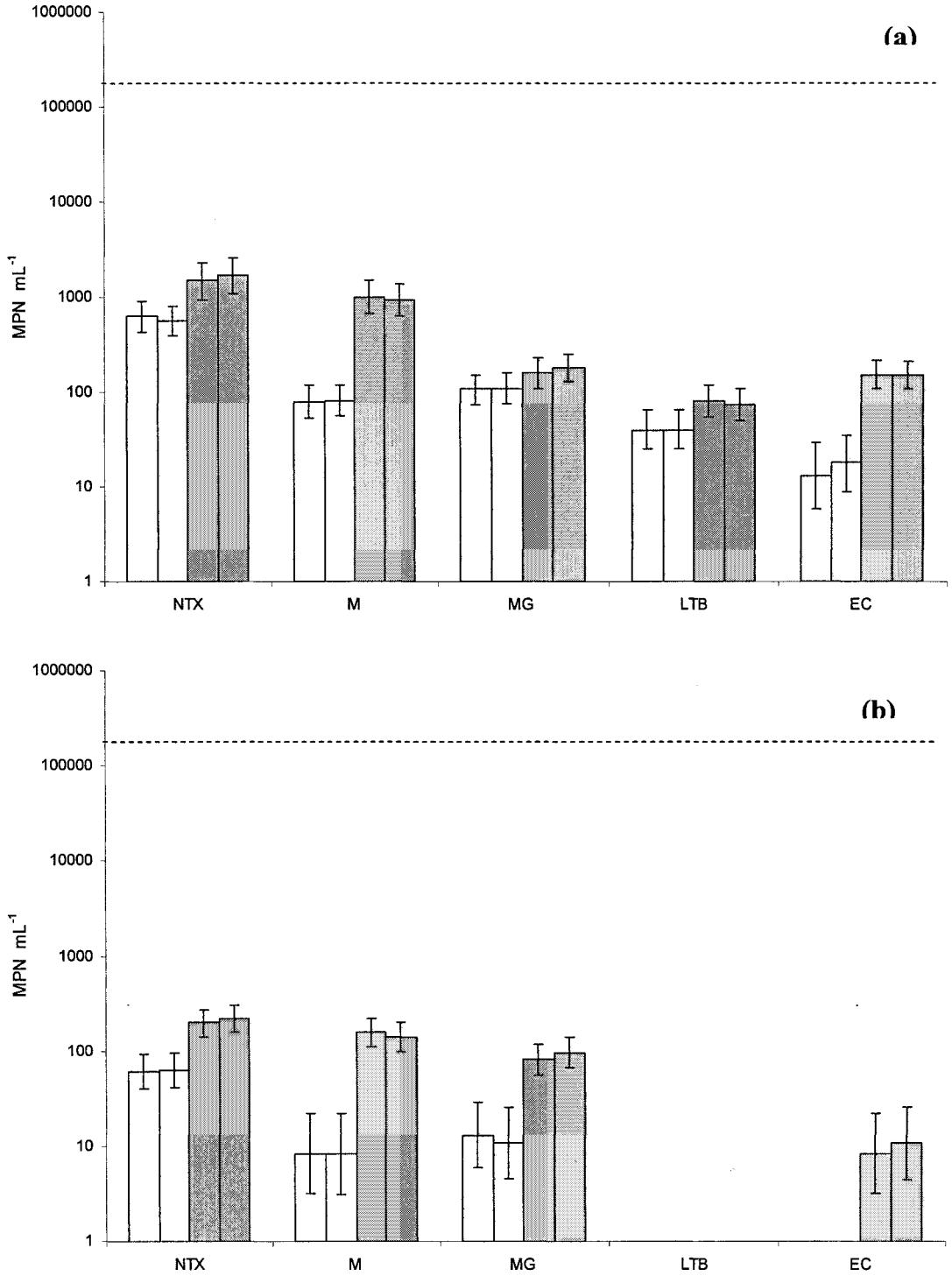


Fig. 6.7 Comparison of novel broth-based medium with standard media for enumerating UV-A exposed *E. coli* NCTC8912 (a) 2 h exposure to UV-A, (b) 3 h exposure to UV-A, under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicates on NTX, MacConkey broth (M), minerals modified glutamate medium (MG), lauryl tryptose broth (LTB), and EC broth (EC). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

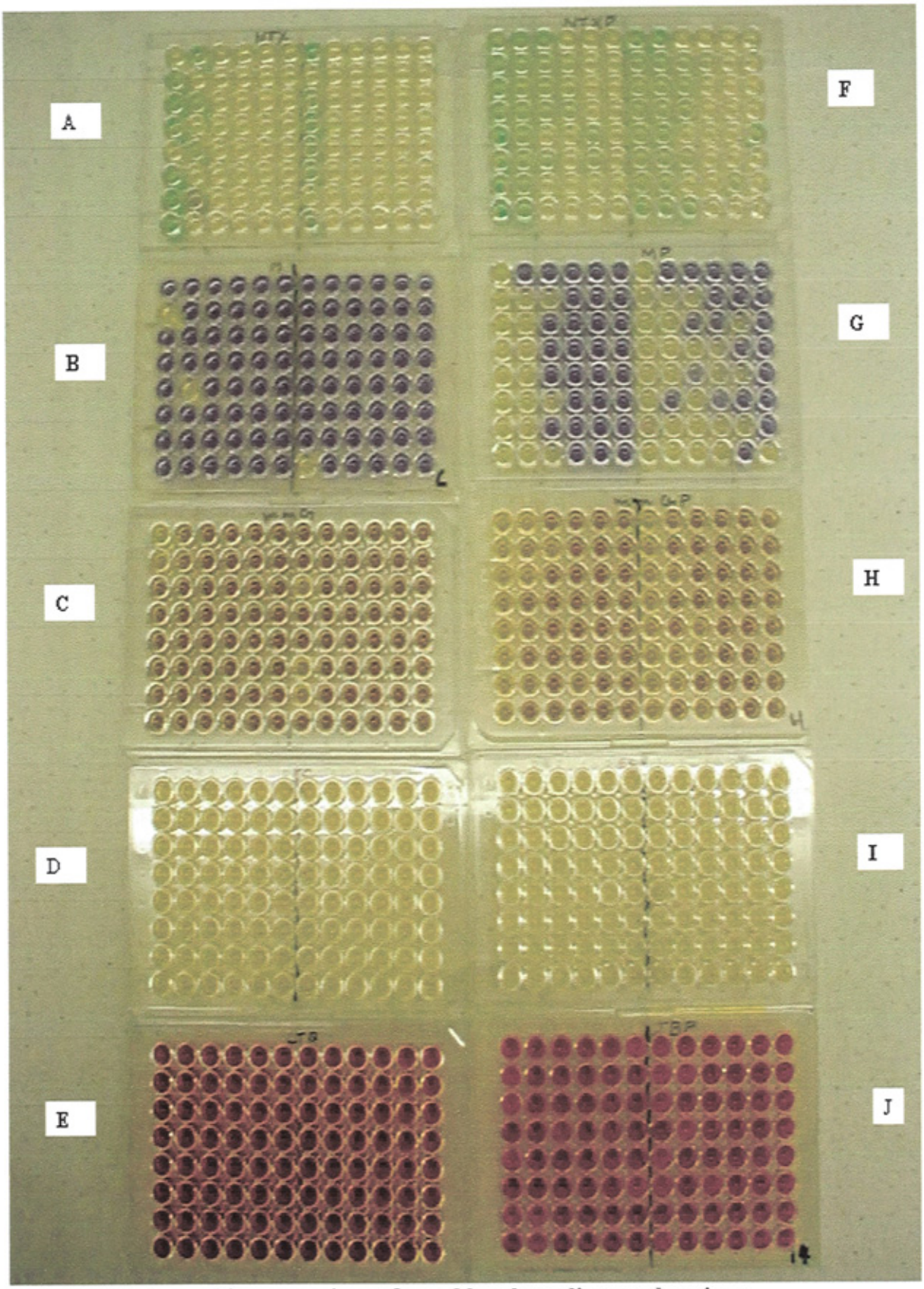


Plate 6.1 Photographic comparison of novel broth medium and various standard media enumerated under aerobic conditions using unsupplemented media: NTX (A), MacConkey broth (B), minerals modified glutamate medium (C), EC broth (D), lauryl tryptose broth (E) and under peroxide-neutralised conditions (growth medium supplemented with 0.05% w/v sodium pyruvate) in NTX (F), MacConkey broth (G), minerals modified glutamate medium (H), EC broth (I) lauryl tryptose broth (J).

6.4 Discussion

The purpose of the experiments described in this chapter was to formulate and develop a broth-based medium for the detection and enumeration of *Escherichia coli* commonly used as a faecal indicator (WHO, 2004a) and subjected to various stressful conditions prevalent in the environment (Fujiioka *et al.*, 1981; Calabrese and Bissonnette, 1990a; Mizunoe *et al.,* 1999; Grey and Steck, 2001; Davies and Mazumder; 2003; Rizzo *et al.*, 2004). The characteristics considered during the formulation of such a medium were: (i) minimum toxic ingredients with a high recovery rate (Bissonnette *et al.*, 1975; Singh and McFeters, 1986; 1990), (ii) a selective component that enables growth of low levels of debilitated bacteria (LeChevallier *et al.*, 1983; McFeters *et al.*, 1986; Calabrese and Bissonnette, 1990b; Fass *et al.*, 1996), (iii) a scavenger of reactive oxygen species, formed due to auto-oxidation of media components or as a by product of respiration induced metabolism, such as sodium pyruvate (Mackey and Derrick, 1986; Sartory, 1995; Czechowicz *et al.,* 1996; Mizunoe *et al.,* 1999; Khaengraeng, 2004; Reed, 2004; Khaengraeng and Reed, 2005). The medium also requires a diagnostic feature, e.g. detection of the enzyme β-glucuronidase by indoxyl –based chromogenic or fluorogenic substrates e.g. 5-bromo-4 chloro-3-indolyl-β-glucuronide or 4-methylumbelliferyl-β-glucuronide (Edberg *et al.,* 1990; Manafi, 2000; Chilvers, 2001; Butterworth *et al.*, 2004) without the need to carry out confirmatory or completed tests that delay the enumeration procedure for up to 3 days (Edberg and Edberg, 1988; Manafi, 1996; 2000).

Thus summarizing, the features of an ideal growth medium should be that it possesses:

(i) sensitivity better than standard selective media used for enumeration of *E. coli* in the UK and US,

(ii) the specificity to enumerate and isolate even one *E. coli* per 100 mL,

(iii) no false-positive or false-negative results,

(iv) no requirement for confirmatory or completed tests, thus providing results from the primary isolation medium,

(v) the ability to resuscitate injured bacteria exposed to sub-lethal environments,

(vi) the capacity to provide results within a 24 h incubation time period,

(vii) no inhibitory components that are major sources of secondary stress to injured bacteria,

Additionally the procedure should be easy to perform and interpret (Edberg *et al.*, 1990; Manafi 1996; 2000; Anon., 2002; Clesceri *et al.*, 1998).

The results in the present study obtained for choice of a selective ingredient in Figures 6.1, 6.2 and 6.3 a-b, demonstrate that initially before sub-lethal injury, *E. coli* cells were in healthy state (Chapter 3; Fig. 3.8) as a similar value per mL were obtained for plate count method and MPN multiwell method under all sets of growth conditions and selective agents. After 2-3 h exposure to UV-A the plate count agar method and MPN multiwell method showed maximum enhancements on the nutrient medium supplemented with or without Tergitol-7 under all sets of growth conditions, with sodium lauryl sulphate incorporated medium showing lowest recovery rate (McFeters *et al.*, 1982; Calabrese and Bissonnette, 1990a; Chilvers, 2001). The results are in parallel agreement with the previous studies (Chapter 4) sodium lauryl sulphate agar gave lowest counts followed by mEndo for the enumeration of sub-lethally injured *E. coli* strains obtained in Chapters 3 and 4.

Tergitol-7 proved to be a selective ingredient that showed a comparable performance to non-selective nutrient agar/broth, whether under aerobic, peroxide-neutralised, anaerobic and ROS-neutralised conditions (Fig. 6.4). This is in agreement with the results obtained by LeChevallier *et al.* (1983) and US Standard Methods (Clesceri *et al.*, 1998), where Tergitol-7 incorporated in m-T7 agar is considered to be effective and resuscitative medium for the growth of stressed bacteria (Chapter 5; Calabrese and Bissonnette, 1990b; Fass *et al.*, 1996). Furthermore, the cells recovered by MPN multiwell method using nutrient broth with and without sodium pyruvate and Tergitol-7 under all three sets of growth conditions were higher than their counterparts for the agar plate count method. These findings support that broth is a better medium for recovery of injured bacteria than the equivalent agar-based medium, which is likely to be due to its lower oxygen content compared to air (Mossel *et al.*, 1980).

The inclusion of DTT (Fig. 6.5) (George *et al.*, 1998; Kirakosyan *et al.,* 2004) or sodium thioglycollate (Fig. 6.6), usually incorporated into media supporting the growth of obligate anaerobes termed as anaerobic agar or thioglycollate agar (Pan and Imlay, 2001), to nutrient broth or agar increased the standard aerobic value using both plate count but MPN multiwell methods and showed minimal increases for peroxide-neutralised, anaerobic or ROS-neutralised conditions (both with and without Tergitol-7) showing that they both are ineffective for further neutralisation of reactive forms of oxygen beyond that achieved with pyruvate alone.

The most widely used defined-enzyme-substrate technology, Autoanalysis Colilert (AC), developed by Edberg *et al.* (1990) is based on the principle of an indicator-nutrient such as *ortho*-nitrophenyl-β-D-galactoside (ONPG) acting as a substrate for the target enzyme which is indicated each time by the release of a chromophore from

the substrate hydrolysed e.g. the release of yellow colour from ONPG by coliforms possessing the enzyme β-galactosidase. The Colilert medium contains an additional fluorogenic substrate, 4-methylumbelliferyl-β- glucuronide (MU-GLUC) which is hydrolysed by the enzyme β-glucuronidase present in *E. coli* that can be detected by a UV-A source such as a hand-held fluorescent light of wavelength 366 nm (Edberg and Edberg, 1988; Edberg *et al.,* 1990; Manafi, 2000). The Colilert medium is available in dehydrated powder and can be used in MPN format, or as a Presence/Absence test (IDEXX Laboratories, Westbrook, Maine). The main drawback of the Colilert test is that can give a weak yellow colour for injured coliform bacteria that may be misread as a negative result (Chilvers, 2001). Francy and Darner (2000) compared the MPN-based Colilert test with membrane filtration, based on a modified mTEC medium (Smith, 1997) and MI agar (Brenner *et al.*, 1993) for comparison of *E. coli* in recreational waters. They reported that MI agar gave the highest correct response percentage and the lowest false-negative percentage in contrast to both mTEC agar and the Colilert test.

In the present study X-GLUC was used as a chromogenic substrate for the specific differention of *E. coli* from other bacteria since after being hydrolysed by the β-glucuronidase enzyme it gives a blue-green colour which is uniformly localised around the bacterial cell, giving an even colour to a broth suspension. This substrate combined with nutrient broth as the basal medium and with Tergitol-7 as a selective ingredient with added sodium pyruvate recovered the highest number of UV-A exposed *E. coli* cells when compared with other selective media such as MacConkey broth, minerals modified glutamate medium, EC broth or lauryl tryptose broth under both aerobic and peroxide-neutralised conditions (Fig. 6.7 a-b). In the next chapter (Chapter 7) this formulation and various other selective media have been used to

process environmental water samples. The formulation is further developed to a format tested under field conditions, in order to evaluate its potential.

Chapter 7

Evaluation of a novel broth-based medium for the enumeration of *Escherichia coli* under field conditions

7.1 Introduction

Modification of the simple multiple tube fermentation or most probable number technique (MPN) into a more economical, sensitive and inexpensive presence-absence (P-A) test was first developed by Clark (1968) to simplify the laborious and time-consuming methods used for isolation, detection and enumeration of coliform bacteria in municipal drinking water supplies (Chilvers, 2001; Clesceri *et al.*, 1998). The P-A test is performed by inoculation of 50 mL of water sample into 50 mL of double strength MacConkey broth modified by adding 10 g of tryptone per litre (Clark, 1968) in a single bottle that allows larger numbers and volumes of samples to be examined in a given time. The US standard method (Clesceri *et al.*, 1998) use a P-A broth that is commercially available in dehydrated form whereas the UK standard method (Anon., 2002) use double-strength minerals modified glutamate medium in a presence-absence test format.

Manja *et al.* (1982) introduced another simple P-A medium for field use, also known as a one tube test, termed the H_2S test for the detection of faecal pollution in drinking water. The H_2S test was based on the detection of hydrogen sulphide-producing organisms and Manja *et al.* (1982) stated that the presence of coliform bacteria is linked with organisms that produce hydrogen sulphide (H_2S). In the H_2S assay, the test medium is added in concentrated form to a tissue paper, which is placed in a McCartney (Universal) bottle, sterilized and dried at 50°C in an oven thereby enabling it to be stored for several months. The water samples to be processed are simply inoculated into these bottles up to a pre-calibrated mark (20 mL) and then allowed to stand at temperatures ranging from 30°C to 37°C. A change of colour of the contents of the bottles from light brown to black indicates the presence of H_2S-producing organisms due to the formation of iron sulphide, typically giving results

within 12-18 hours (Pillai *et al.*, 1999; Nair *et al.*, 2001). Modifications of the H_2S test differ in the medium composition, the preparation of the medium and supporting materials, the test format and sample volumes, incubation time, incubation temperature and scoring of results (e.g. WHO 2002b). In addition, other researchers have tried to identify the bacteria present in positive H_2S test bottles; for example, Castillo *et al.,* (1994) isolated bacteria belonging to the family Enterobacteriaceae and also *Clostridium perfringens* in samples scoring as positive in the H_2S test, stating that this medium scored 10% more positives than other coliform tests and was suitable for screening tropical and subtropical potable waters. Ratto *et al.* (1989) identified *Citrobacter* to be a common bacterium in positive H_2S test samples and evaluated this medium in comparison to other MPN and P-A tests commonly used for isolation of total coliforms and faecal coliforms at incubation temperatures of 22°C and 35°C. Their research concluded the H_2S test medium to be equally sensitive to other coliform tests and recommended its use in places where laboratory facilities are not available. Kaspar *et al.*, (1992) evaluated and tested the H_2S approach modified by lyophilizing the medium using no tissue paper rather than for screening different water sources and concluded the test to be useful for treated or piped water and unsuitable for surface waters and dug well water. Venkobachar *et al.*, (1994) modified the original H_2S test medium by including L-cystine (utilized by H_2S-producing organisms and broken down to release H_2S) and used the medium in five 20-mL bottles in an MPN format rather than a P-A format. They compared the original H_2S test medium with the H_2S test medium containing L-cystine and stated on the basis of correlation analysis that the latter was more sensitive than the original test medium. Nagaraju and Sastri (1999) used the standard H_2S test medium for assessing ground water wells in Mysore, India while Pillai *et al.* (1999) evaluated and used different modifications of the original H_2S test medium for the detection of

faecal contamination, using distilled water seeded with diluted faeces in 100-mL volumes, incorporating L-cystine and higher incubation temperatures, ranging between 28°C to 44°C. Rijal *et al.* (2000) used this test medium in two versions, firstly a MPN method consisting of replicate sample volumes of 1mL to 100 mL and secondly in a membrane filter agar medium format to enumerate total coliforms and *E. coli* in ground water and stream water samples. They regarded this test in both formats to be ideal for testing the hygienic quality of water. Manja *et al.* (2001) compared the original test medium with modifications e.g. by incorporating L-cystine, using various sample volumes, incubation temperatures and incubation times with standard MPN method used for testing coliform bacteria. They suggested that the H_2S test with L-cystine supplementation gave better results than the original formulation. All of these researchers suggested that although the positive H_2S test organisms may not always be coliforms, they may be organisms typically found within the intestines of warm-blooded animals and therefore can indicate faecal contamination. Furthermore, the H_2S test medium was demonstrated to be simple, needing minimum resources and time, of low cost to prepare, requiring minimum laboratory support and personnel and therefore well-suited for the routine bacteriological quality assessment of water sources under field conditions in rural areas.

The presence-absence tests discussed above are not quantitative and would also still require additional steps for full confirmation of presumptive test results. More recently, microbiologists have investigated the need for exemption of these steps by incorporation of a chromogen for the enzyme β-glucuronidase such as 5-bromo-4 chloro-3-indolyl-β-glucuronide (X-GLUC) or a fluorogen such as 4-methylumbelliferyl-β-glucuronide (MU-GLUC) into primary isolation media as an

alternative to using confirmation steps in traditional liquid and agar-based media (Berg, 1988; Alonso *et al* 1996, 1999; Brenner, 1996; Budnick, 1996; Manafi *et al* 1996; Rhodes 1997; Geissler 2000; Manafi, 2000). Many commercial chromogenic and/or fluorogenic products such as Colilert (IDEXX), Colifast (Colifast AS), Coliquick (Hach), Colisure (Millipore) and m-Coliblue 24 broth are used, either in presence-absence or MPN format for the detection of coliforms from drinking water; they have excellent specificity and sensitivity and give results within 18-24 hours (Edberg *et al.,* 1988, 1990; Grant, 1997; Samset *et al.,* 2000). Another substrate, 8 hydroxyquinoline-β-glucuronide (8-HQ-GLUC), is a less commonly used chromogenic agent that could be incorporated into the basal growth medium along with a ferric salt such as ferric ammonium citrate (James and Yeoman, 1988; James *et al.*, 2000a). This substrate hydrolyses in the presence of β-glucuronidase-positive strains such as *Escherichia coli* to produce a black-coloured reaction due to the formation of an iron-8-hydroxyquinoline complex (James *et al.,* 1996; Reinders *et al.*, 2000b; James *et al.*, 2001; Butterworth *et al.*, 2004). A commercially available medium, Uricult Trio® (Orion Diagnostica, Espoo, Finland), contains 8-HQ-GLUC and is used for the detection and isolation of *E. coli* strains in urine samples (Dalet and Segovia, 1995; Larinkari and Rautio, 1995). This Chapter describes experiments carried out to develop and test a broth-based medium for the detection and enumeration of *E. coli* based on the chromogenic substrate 8-HQ-GLUC, which turns the test broth black as a "positive" test result in a multiple tube format as an alternative to the single tube H_2S test (Manja *et al.*, 1982).

The specific objectives of the present study were:

1. To develop and optimize a broth-based field medium based on 8 hydroxy quinoline-β-glucuronide (Coliblack) using MPN multiwell assay under aerobic conditions with and without sodium pyruvate supplementation in the growth medium and to compare it with various other differential selective media used for the enumeration of *E. coli*.
2. To compare the novel broth-based field medium in four bottle MPN format against the H_2S medium, i.e. "Coliblack-12" and "H_2S-12" test-medium with a plate counting method using Chromocult agar.
3. To evaluate under field conditions the efficacy of "Coliblack-4" bottle test with the help of an instructional sheet given to unskilled personnel for testing their drinking water through the use of a feedback questionnaire.

7. 2 Materials and Methods

Various water samples from the River Tyne in Newcastle, U.K, and in Panjab, India (maps in Chapter 2) were used for evaluation of the field medium. The broad methodology and procedures common to this Chapter are discussed below.

7.2.1 Comparison of the novel broth-based media with standard media

A total of 10 water samples were processed using the MPN multiwell assay in duplicates as described in detail in Chapter 2. The first three samples (River Tyne) were processed in Northumbria University, Newcastle against a broad range of media, while remaining seven samples were processed in Panjab University, Chandigarh against selected media from first three samples. The formulated media included NTX (see Chapter 6), TTX, TTM (see below), minerals modified glutamate medium, lauryl tryptose broth and H_2S test broth medium, with and without 0.05% sodium pyruvate incorporation with aerobic incubation at 37°C for 48 h. The individual ingredients of those media which have not been described in Chapter 6 were prepared as follows at two and half times normal strength, added to 100 mL distilled water in the amounts described below:

Basal medium: The basal medium was chosen from individual ingredients of membrane lauryl tryptose broth (Clesceri *et al.*, 1998) after removing phenol red and sodium lauryl sulphate; this was chosen as it is a standard medium for coliforms in the US. The basal medium consisted of 5 g of tryptose, 1.25 g of sodium chloride, 0.688 g of dipotassium hydrogen phosphate, 0.688 g of potassium dihydrogen phosphate, 2.5mL of tergitol -7 from pre-prepared stock (0.94 mL in 100mL sterile water) to give 0.025 g, 1.25 g of lactose.

- **TTX (tryptose, tergitol, X-GLUC):** basal medium + 250 µL of X-GLUC stock solution (50 mg in 1 mL).

- **TTM (tryptose, tergitol, MU-GLUC):** basal medium + 250 µL of MU-GLUC stock solution (50 mg in 1 mL).

- **H_2S test medium:** 5 g of peptone, 0.375 g of di-potassium hydrogen phosphate, 0.1875 g of ferric ammonium citrate, 0.25 g of sodium thiosulphate, 0.05 g of sodium lauryl sulfate.

The test-positive MPN values obtained using the various broth media were confirmed as positive for *E. coli* MPN values after streaking each test-positive well on Chromocult agar. Representative purple colonies were examined by performing an indole test, oxidase test and Gram staining. Thus purple-coloured β-glucuronidase-positive and β-galactosidase-positive colonies on Chromocult agar, with the production of indole from tryptophan (based on the formation of a deep red colour in tryptone water after 24 hour incubation on addition of Kovac's reagent), with no appearance of a deep blue purple colour on the oxidase test strip (oxidase-negative) and Gram-negative rods were confirmed as positive *Escherichia coli*. With the H_2S-test medium the growth-positive (test-negative) values were also confirmed because the test-positive presumptive values alone might give false-negative results leading to an underestimation of the true count.

7.2.2 Development of "Coliblack" field medium and its comparison with TTM

A single tube format was used for the development of the novel broth-based medium. The amount of 8-HQ-GLUC and iron needed in the basal medium was established by testing different combinations of both ingredients in single-strength medium. The concentrations of 8-HQ-GLUC (50 mg L^{-1} to 200 mg L^{-1}) with two ferric salts, i.e. firstly ferric ammonium sulphate (0.25 g L^{-1} to 1.0 g L^{-1}) in Table 7.1 and secondly ferric ammonium citrate (0.125 g L^{-1} to 1.0 g L^{-1}) in Table 7.2 were added separately to basal medium as described in tables below:

Table 7.1 Different combination of 8-hydroxyquinoline-glucuronide and ferric ammonium sulphate in the basal medium used for the optimisation.

8-hydroxyquinoline glucuronide (8-HQ-GLUC mg L^{-1})	**ferric ammonium sulphate (FAS g L^{-1})**
50	0.25
100	0.50
150	0.75
200	1.0
150	1.0
100	1.0
50	1.0

Table 7.2 Different combination of 8-hydroxyquinoline-glucuronide and ferric ammonium citrate in the basal medium used for the optimisation.

8-hydroxyquinoline glucuronide (8-HQ-GLUC mg L^{-1})	**ferric ammonium citrate (FAC g L^{-1})**
50	0.125
100	0.25
150	0.375
200	0.50
200	1.0
150	1.0
100	1.0
50	1.0

The media with various concentrations of 8-HQ-GLUC and ferric salts in the basal medium were inoculated with *E. coli* to visually check the amount of black colouration in each bottle. To establish the most optimum concentration of the 8-HQ-GLUC and ferric salts in each test-positive bottle (black colouration), the absorbance of each test-positive bottles with different combinations of the 8-HQ-GLUC and ferric ammonium citrate incorporated in the basal broth medium were measured at 550 nm by spectrophotometer (CECIL1000 Series, Cheadle, Chershire, UK). The concentration of 8-HQ-GLUC and ferric salt in basal medium giving maximum absorbance was selected for further experimentation.

A total of 10 water samples were processed using the MPN multiwell assay to compare MPN values for *E. coli* that were obtained in a medium containing 8-HQ-GLUC plus ferric ammonium citrate (FAC) with a fluorogen MU-GLUC (100 mg L^{-1}) in TTM with 0.05% w/v sodium pyruvate. The substrates were added in the basal medium, autoclaved and inoculated with *E. coli*. The MPN multiwell trays for each substrate were incubated at 25°C and 37°C. In all subsequent experiments this combination of 8-HQ-GLUC and selected iron salt has been termed "Coliblack".

7.2.3 Comparison of "Coliblack-12" and "H_2S-12" with a plate counting method using Chromocult agar

The ingredients in Coliblack medium (basal medium + 8-HQ-GLUC + FAC + pyruvate) and H_2S medium were prepared at twenty times the normal strength, added to distilled water. The 1 mL of 20-strength broth was then dried on filter paper prior to autoclaving (Manja *et al.*, 1982; Manja *et al.*, 2001). In the test, four dilution bottles, i.e. bottles A, B, C and D (see Plate 7.1 for further details) tested in triplicate format, i.e. A_1, A_2 and A_3 etc were used for "Coliblack-12" and "H_2S-12". Whereas

in the plate counting method triplicate sets of Chromocult agar plates were used (see Chapter 2 for composition). The "Coliblack-12" and "H_2S-12"bottles were marked with permanent marker with two lines that were at the volume of 22 mL and 20 mL for all bottles four bottles in triplicate. The bottle constituting series A were filled up to the upper mark with water sample, mixed carefully by inversion for at least six times while the bottles constituting series B, C and D were filled up to the lower mark with sterile water. A volume of 2 mL of the sample from the A series bottle was poured carefully into B series bottle until the level in B reached the upper mark. Then the B series bottles were mixed carefully by inversion for at least six times. Similarly the other two dilutions were carried out for bottles of C and D series. In the case of Chromocult agar the samples were spread plated or membrane-filtered, depending upon the expected number of bacteria in the sample. All the growth media were incubated at 37°C for 24 hours. The MPN values per mL with upper and lower 95% confidence limits was calculated for broth media using the MPN calculator (Curiale, 2004) while for Chromocult agar the plate count CFU per mL was calculated as described in Chapter 2. The MPN values were calculated for firstly test-positive wells and secondly for confirmed *E. coli* wells. In all subsequent experiments laboratory evaluation of the 4 bottle Coliblack field medium will be termed "Coliblack-4", whereas the 12 bottle Coliblack field medium will be termed "Coliblack-12" and the 12 bottle H_2S test medium will be termed as "H_2S-12".

Plate 7.1 Photograph of four bottle novel broth field-based medium termed "Coliblack 4" with the bottles A, B, C and D constituting a set, where undiluted sample is added to bottle A to the upper mark while mineral water is added to bottles B, C and D to the lower mark. The 2 mL of water from A is then transferred to bottle B till it reaches the upper mark and so on to bottles C and D in turn.

7.2.4 Field evaluation of "Coliblack-4"

The Coliblack-4 bottles were prepared as described in section 7.2.3 and given for evaluation to 12 unskilled personnel belonging to rural localities in Panjab, India for a preliminary field evaluation of the method. The evaluation consisted of an examination of a single water sample using a prepared instructional sheet, giving detailed steps for conducting the test and providing feedback from the persons taking part as described in next page.

Instruction sheet

Four Coliblack bottles are provided to you, labelled A, B, C and D for water testing.

1. Take the water sample and fill bottle A to the UPPER MARK carefully and mix slowly to avoid bubbles for a total of six times.
2. Fill the other three bottles B, C and D to the LOWER MARK with the MINERAL WATER provided to you.
3. Carefully pour a small amount of sample from bottle A into bottle B until the level in bottle B reaches the UPPER MARK and mix for six times.
4. In the same manner pour sample from bottle B to C mix and then C to D; mix for six times after each step. Note the initial colour/appearance.
5. Keep all four bottles in a cupboard for 48 h.
6. Check the bottles for black colouration either on the filter paper or in the bottle. Score it as positive, or if no colouration and if the colour of the bottle is light brown score it as negative.
7. Note down the number of positive bottles (those which have changed colour from light brown to BLACK) and by referring to the table below for interpretation.

EXAMPLE RESULTS

Number of positive bottles:	Description of level of contamination with faecal bacteria:
0	**No detectable contamination**
1	**Slightly contaminated (MPN = 0.12 per mL)**
2	**Moderately contaminated (MPN = 1.30 per mL)**
3	**Highly contaminated (MPN = 14.0 per mL)**
4	**Very Highly contaminated (MPN = > 14.0 per mL)**

YOUR RESULTS

Number of positive bottles:	Description of level of contamination with faecal bacteria:
0	
1	
2	
3	
4	

Feedback questionnaire for participants

(a) Kindly circle the most relevant answer:

1. Did you get any positive results with the test bottles? YES/NO
2. Did you find the test easy to use? YES/NO
3. Could you follow the instructions easily? YES/NO
4. Could you clearly 'read' the result as either positive or negative? YES/NO
5. Could you easily interpret results using the example table? YES/NO

(b) Kindly give your opinions/comments about the test in a few words:

1. Did you have any specific problems while performing the test? (please explain briefly)

2. Was any part of the instruction sheet difficult to follow? (please explain briefly)

3. Did you get results in the stated 48 h period? (did you check after shorter or longer times?)

4. What is your understanding of a positive outcome of the test (a black tube/precipitate)?

5. Do you have any other comments, opinions, or suggestions?

7.3 Results

7.3.1 Comparison of the novel broth-based media with standard media

Figure 7.1a displays presumptive MPN value per mL obtained for a sample of River Tyne water, enumerated using the multiwell format under aerobic conditions with and without pyruvate supplementation for novel broth-based media in duplicates using either (i) NTX, or (ii) TTX, or (iii) TTM and several broth media currently used for water quality testing, namely MacConkey broth, minerals modified glutamate medium, lauryl tryptose broth, and H_2S test medium. The presumptive results show similar MPN values on the three novel field media with and without pyruvate supplementation. For the conventional standard media with and without pyruvate supplementation, MacConkey broth gave the highest MPN value compared with the other media, with lauryl tryptose broth and minerals modified glutamate medium giving intermediate MPN values, with the three novel field media being slightly lower than these three and the lowest MPN value obtained on the H_2S test medium.

Figure 7.1b displays results for each medium as (test-positive wells only) MPN values after confirmation of *Escherichia coli.* The percentage of confirmed MPN values (number of test positive value confirming as positive for *E. coli*) for the three novel media (NTX, TTX, TTM) with and without pyruvate supplementation confirmed at almost 99% test-positive, 89% by H_2S test medium, 52% by minerals modified glutamate medium, while MacConkey and lauryl sulphate broth confirmed at around 40% of the presumptive MPN.

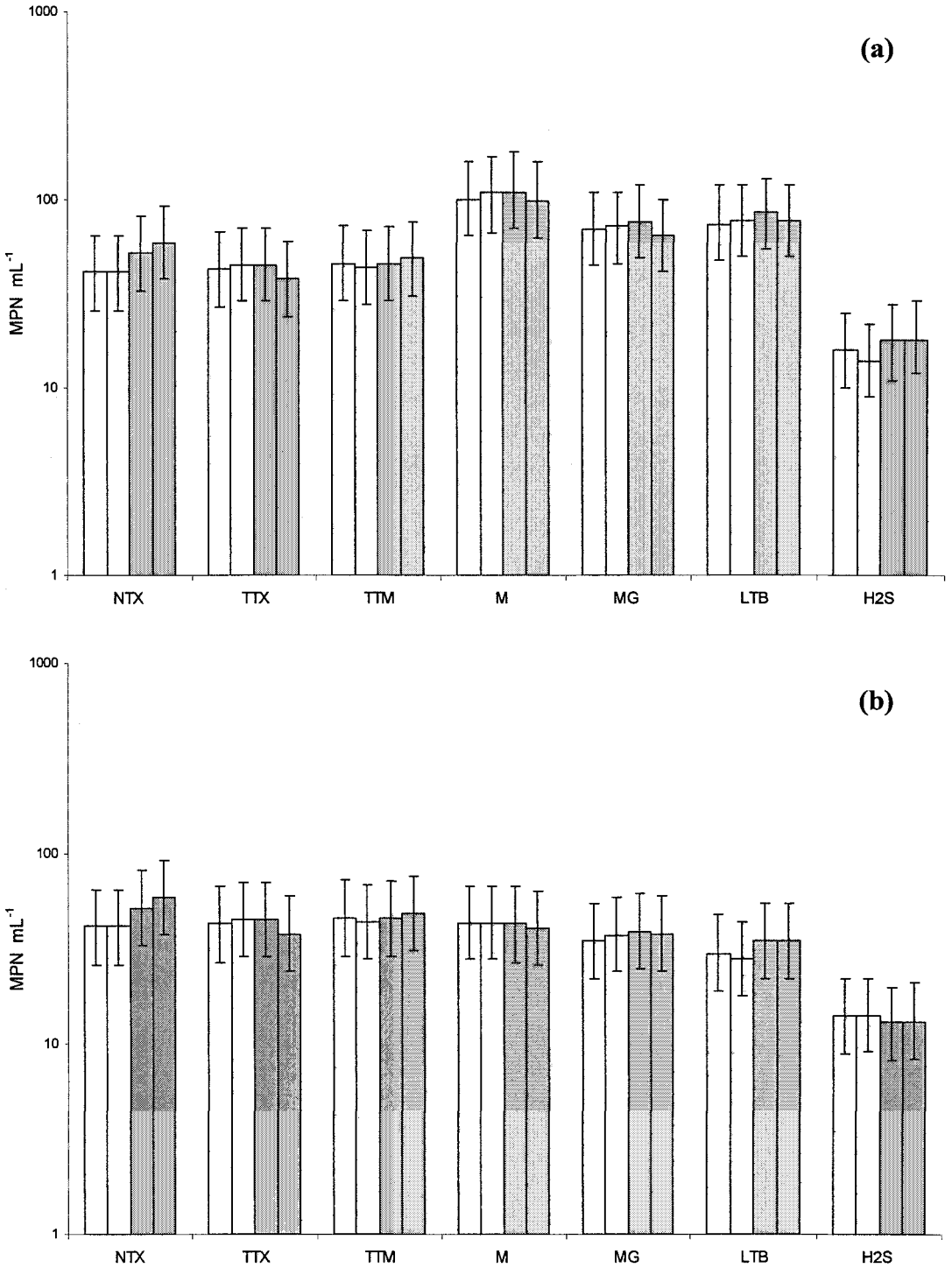


Fig. 7.1 Comparison of novel broth-based media with standard media using River Tyne water sample 1 (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on NTX, TTX, TTM, MacConkey broth (M), minerals modified glutamate medium (MG), lauryl tryptose broth (LTB), and H_2S test medium (H2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

As an example to explain the processing of results obtained in lauryl sulphate broth (false-positives at presumptive stage) with 0.05% sodium pyruvate supplementation using MPN multiwell trays (from Fig. 7.1)

Number of growth-positive presumptive MPN wells = 300 MPN mL^{-1}

Number of test-positive presumptive MPN wells = 160 MPN mL^{-1}

Number of growth-positive confirmed MPN wells = 36 MPN mL^{-1}

Number of test-positive confirmed MPN wells = 36 MPN mL^{-1}

Thus lauryl sulphate broth gave highest number of false-positive results (yellow coloured wells) as many of the wells were test-positive at the presumptive stage but were not confirmed as *E. coli*.

In the case of the H_2S test medium all wells positive for growth were used to provide a growth-positive presumptive MPN mL^{-1}. The wells turning black after the incubation time were termed as presumptive test-positive. All growth-positive and test-positive wells were tested at the confirmatory stage for *E. coli* (Fig. 7.2). An example to explain the results obtained in the H_2S test medium (false-negative) with 0.05% sodium pyruvate supplementation using MPN multiwell plates (Fig. 7.1)

Number of growth-positive presumptive MPN wells = 330 MPN mL^{-1}

Number of test-positive presumptive MPN wells = 28 MPN mL^{-1}

Number of growth-positive confirmed MPN wells = 43 MPN mL^{-1}

Number of test-positive confirmed MPN wells = 23 MPN mL^{-1}

The H_2S test medium thus gave the highest number of false-negative results (no black colouration in wells) as the wells were growth-positive but test-negative at the presumptive stage but then confirmed as *E. coli*.

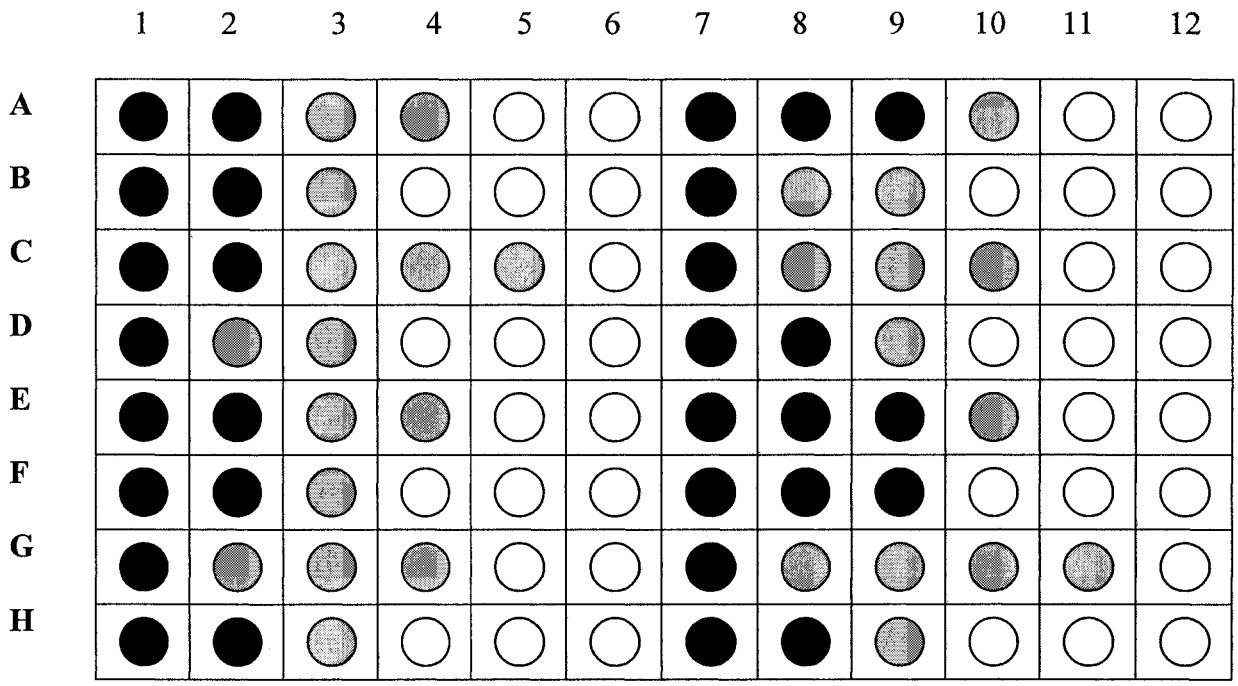


Fig. 7.2 Diagrammatic representation of 96-well multiwell tray with 12 x 8 wells used for preparing five serial four-fold dilutions with 16 replicates constituting duplicate columns for each dilution (2 x 8), used for enumerating *E. coli* (confirmatory tests) from H_2S test medium. Here the black circles represent test-positive wells, the grey circles + black circles represent growth-positive wells and the unshaded circles represents growth-negative wells.

Figure 7.3a-b and 7.4a-b provides presumptive and confirmed MPN values per mL for two further samples of River Tyne water taken on different occasions, showing similar overall trend as were obtained in Figure 7.1a-b. The novel broth based media, i.e. NTX, TTX, TTM confirmed the highest number of presumptive test-positive MPN values followed by MacConkey, minerals modified glutamate medium, lauryl sulphate broth, while the H_2S test medium gave the highest number of false-negative results and the lowest score for test-positive *E. coli* MPN values.

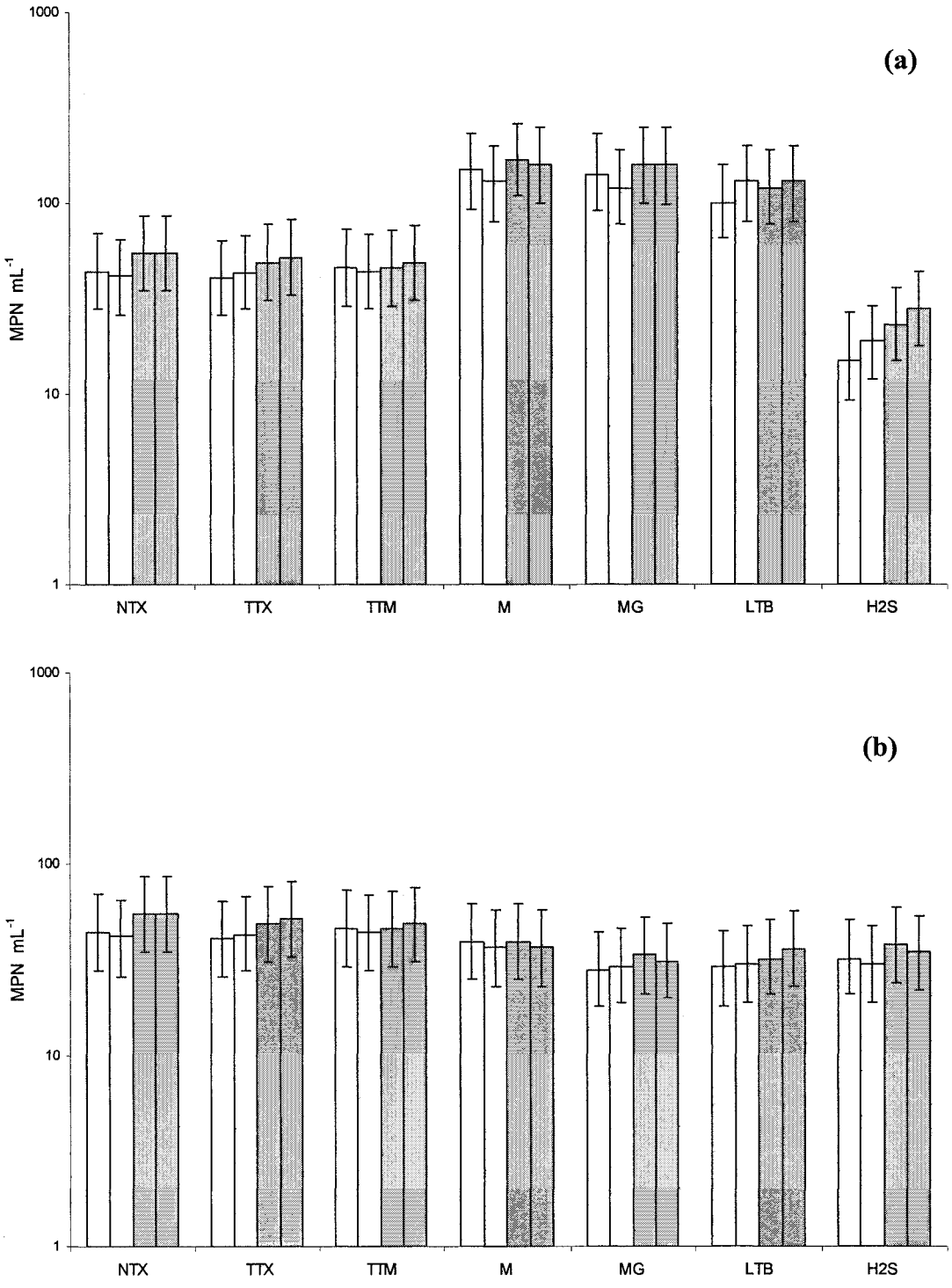


Fig. 7.3 Comparison of novel broth-based media with standard media using River Tyne water sample 2 (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on NTX, TTX, TTM, MacConkey broth (M), minerals modified glutamate medium (MG), lauryl tryptose broth (LTB), and H_2S test medium (H2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

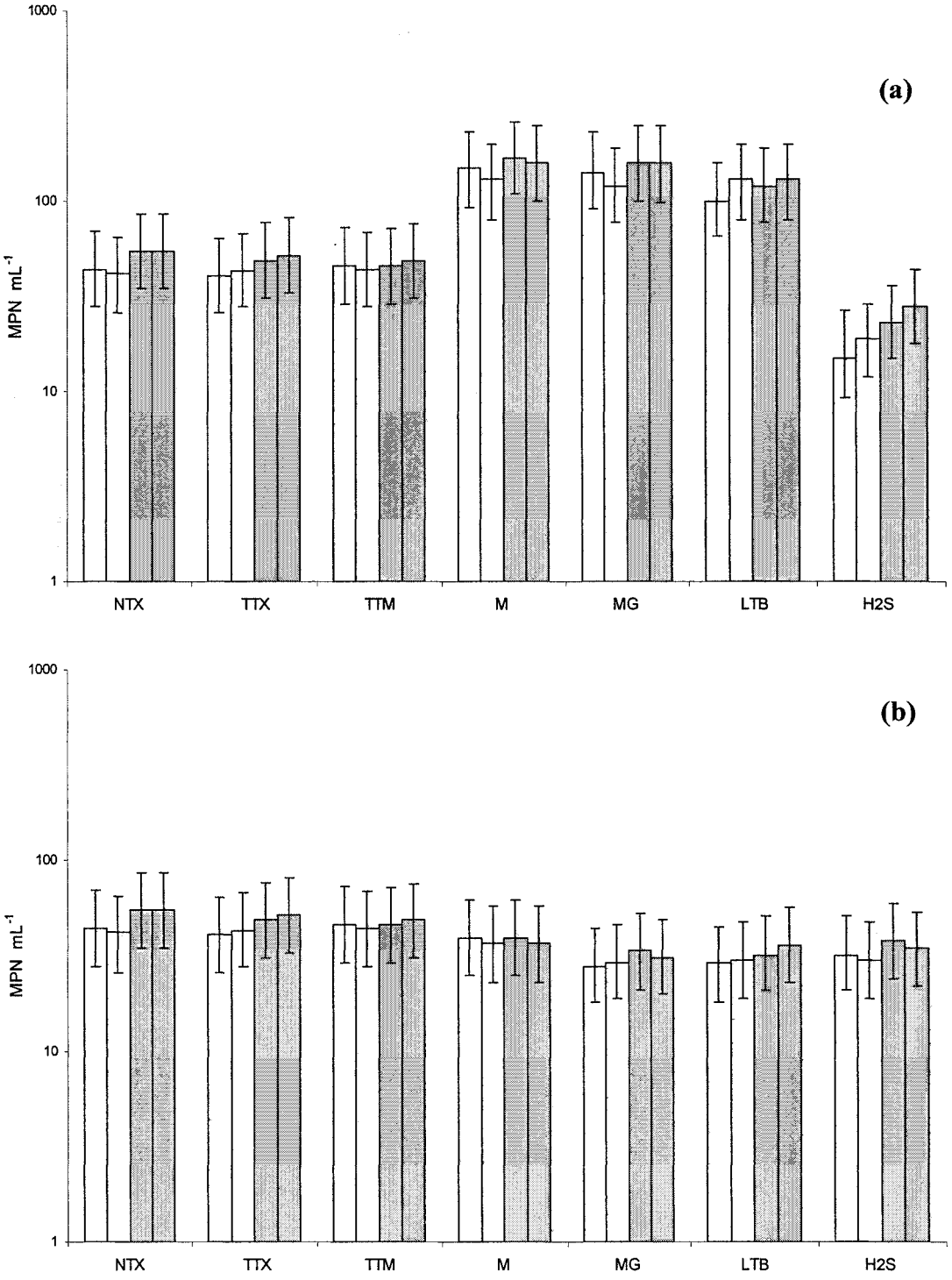


Fig. 7.4 Comparison of novel broth-based media with standard media using River Tyne water sample 3 (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on NTX, TTX, TTM, MacConkey broth (M), minerals modified glutamate medium (MG), lauryl tryptose broth (LTB), and H_2S test medium (H2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

The results for seven water samples processed in India using a narrower range of media namely TTM, MacConkey broth, lauryl sulphate broth and H_2S test medium, all prepared with and without 0.05% sodium pyruvate supplementation in duplicates are shown in Figures 7.5 to 7.11 for presumptive test positive MPN values and secondly for confirmed *E. coli* MPN values. The presumptive results (Fig. 7.5a-7.11a) showed broadly the same pattern as was obtained in Fig. 7.1a with MacConkey and lauryl sulphate broth with and without pyruvate supplementation giving highest MPN values followed by TTM, while the H_2S test medium gave lowest MPN values. The test-positive confirmed results gave maximum values for TTM, at close to 100% (Figs. 7.5b-7.11b) followed by MacConkey broth and lauryl sulphate broth, while the H_2S test medium gave the lowest confirmed MPN values (growth positive) for *E. coli* MPN. Despite the fact that the error bars were overlapping while calculating the MPN value for each media, the results show substantial differences in duplicate multiwell trays, in which each value obtained is in agreement with the other value across various growth media. The inclusion of sodium pyruvate in various selective media increased the MPN value in many cases, but not sufficient to be statistically significant.

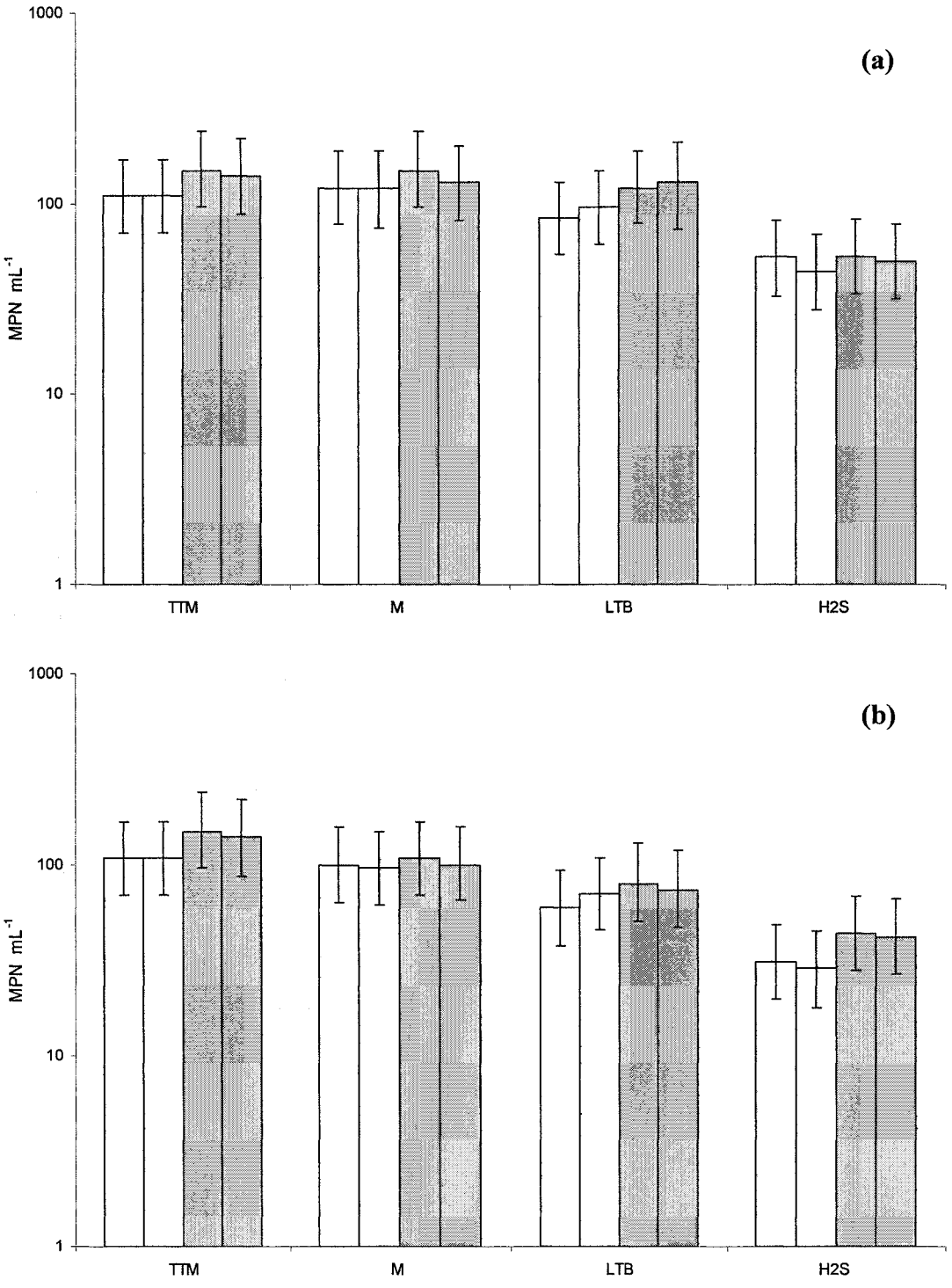


Fig. 7.5 Comparison of novel broth-based media with standard media using PGI water sample in India (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on TTM, MacConkey broth (M), lauryl tryptose broth (LTB), and H_2S test medium (H_2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

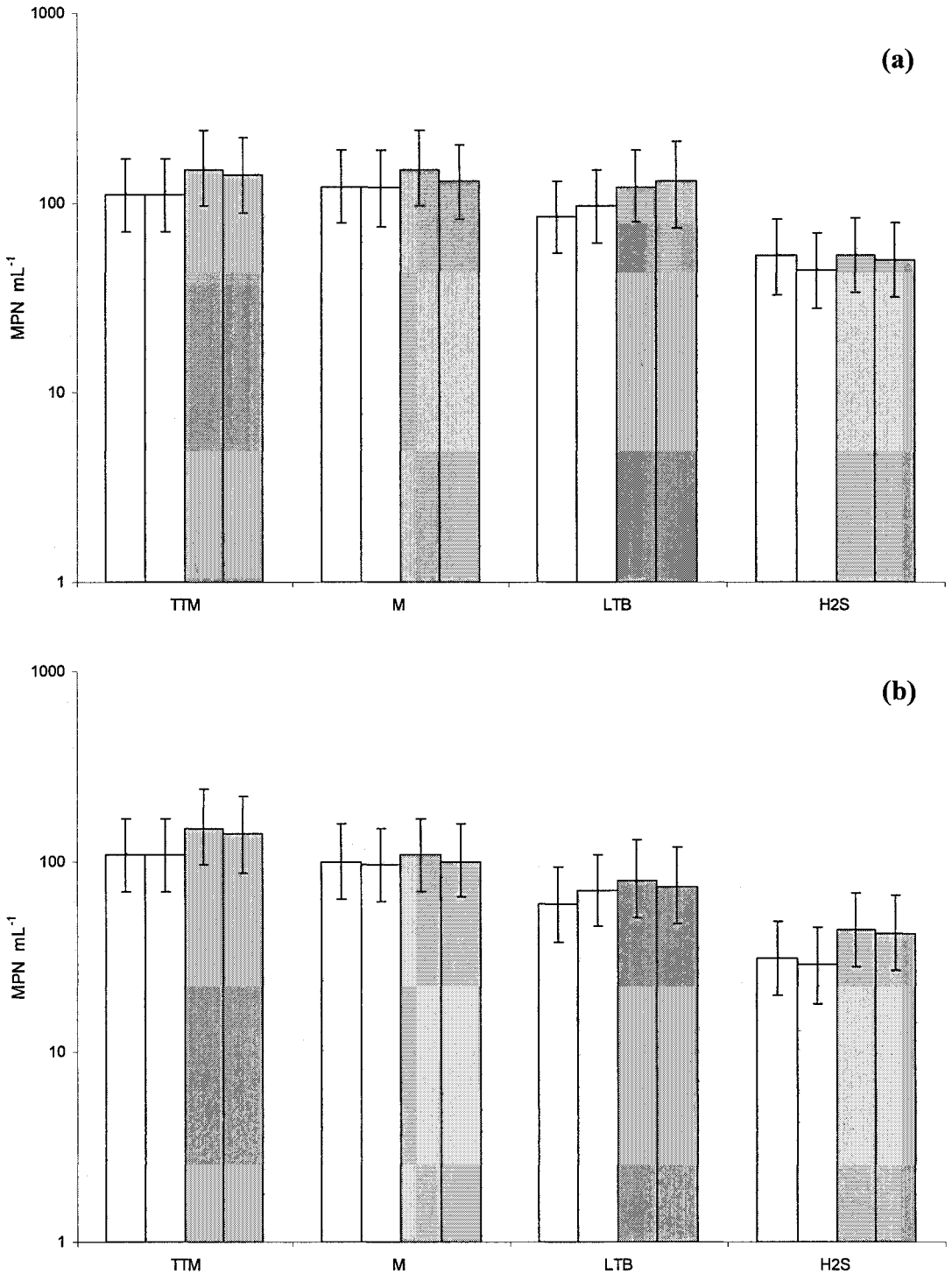


Fig. 7.6 Comparison of novel broth-based media with standard media using Doraha River sample in India (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on TTM, MacConkey broth (M), lauryl tryptose broth (LTB), and H_2S test medium (H_2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

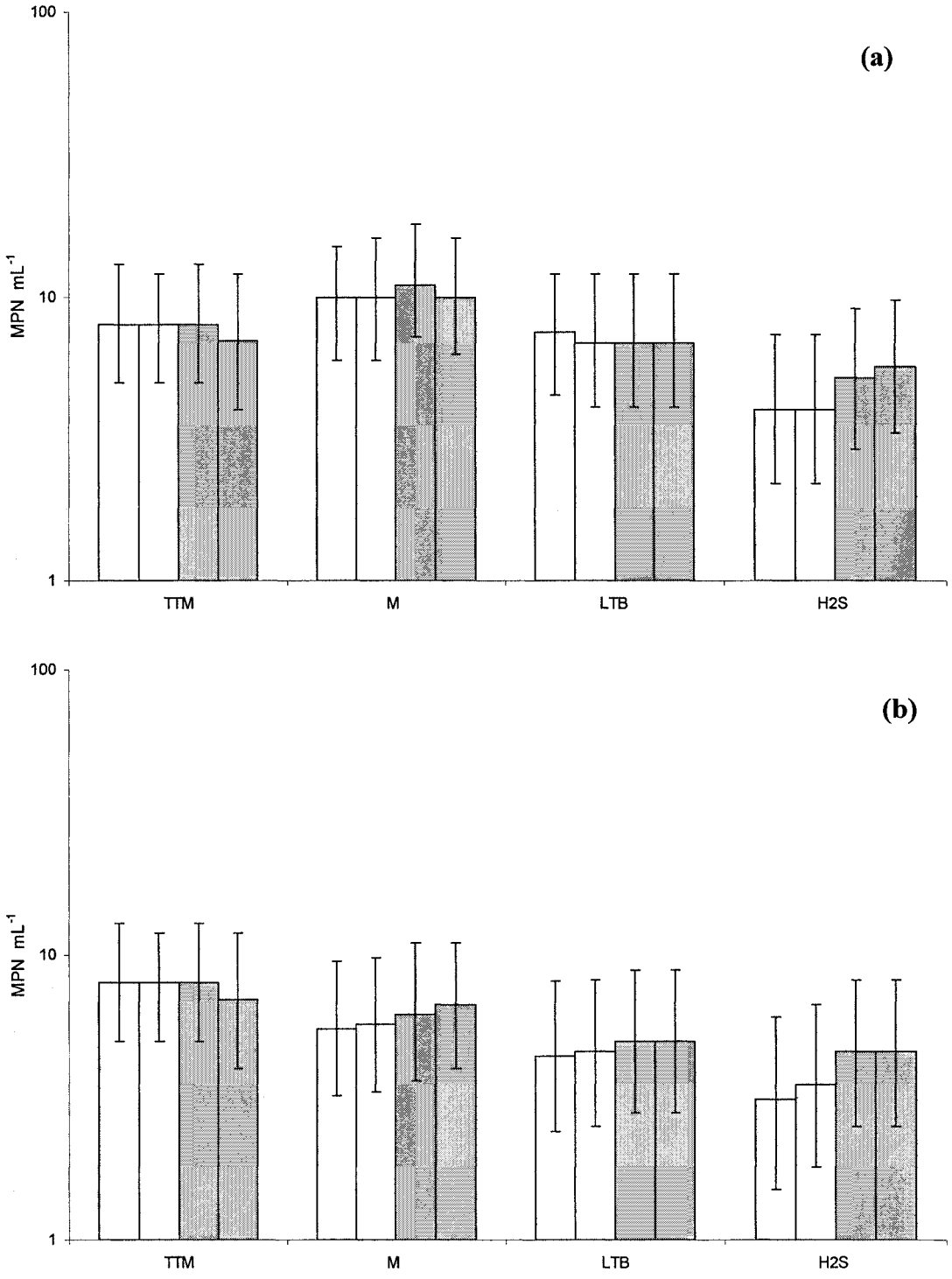


Fig. 7.7 Comparison of novel broth-based media with standard media using Khamanon River sample in India (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on TTM, MacConkey broth (M), lauryl tryptose broth (LTB), and H_2S test medium (H_2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

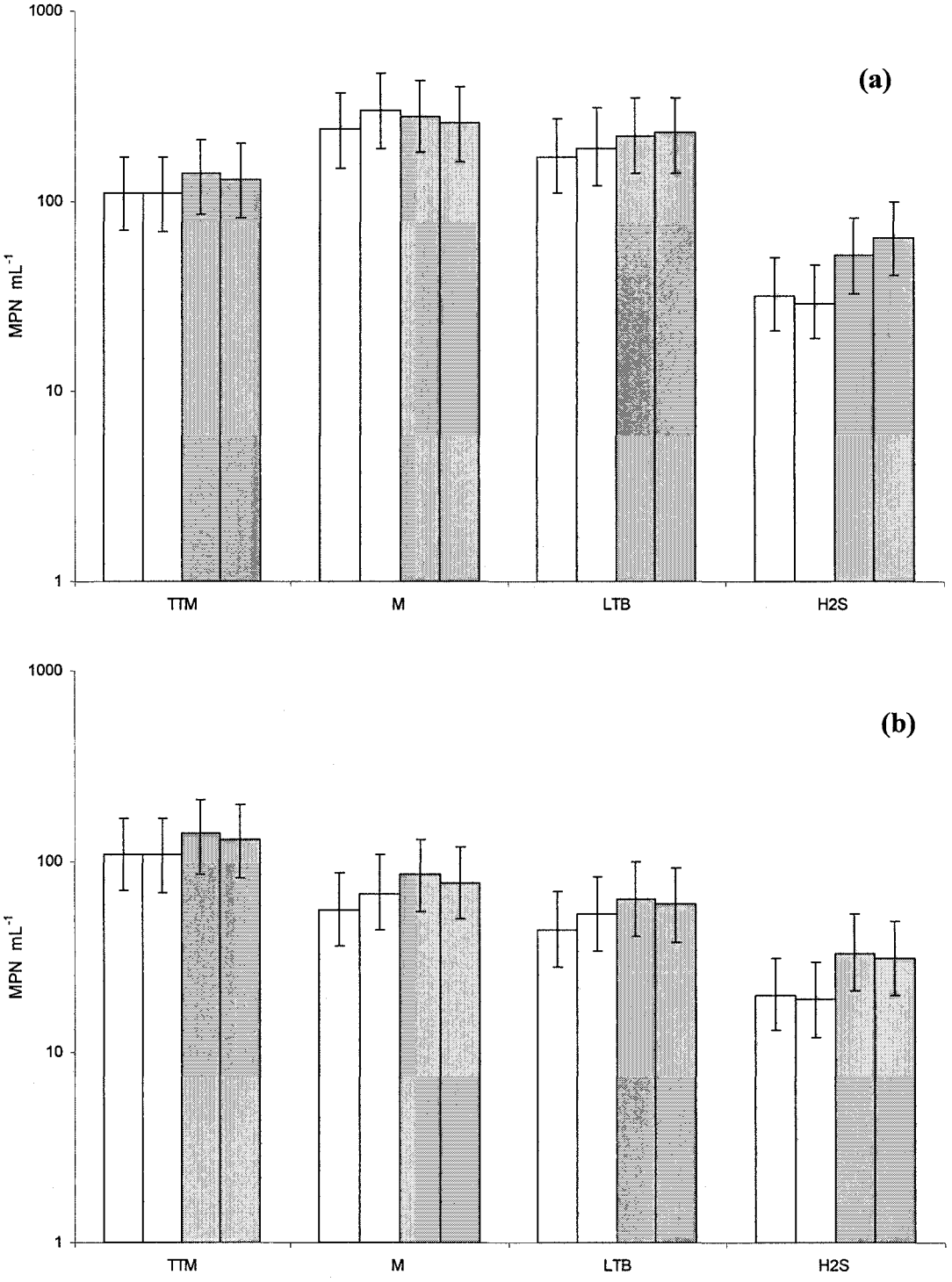


Fig. 7.8 Comparison of novel broth-based media with standard media using sewage contaminated water sample from PGI block-I in India (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on TTM, MacConkey broth (M), lauryl tryptose broth (LTB), and H_2S test medium (H_2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

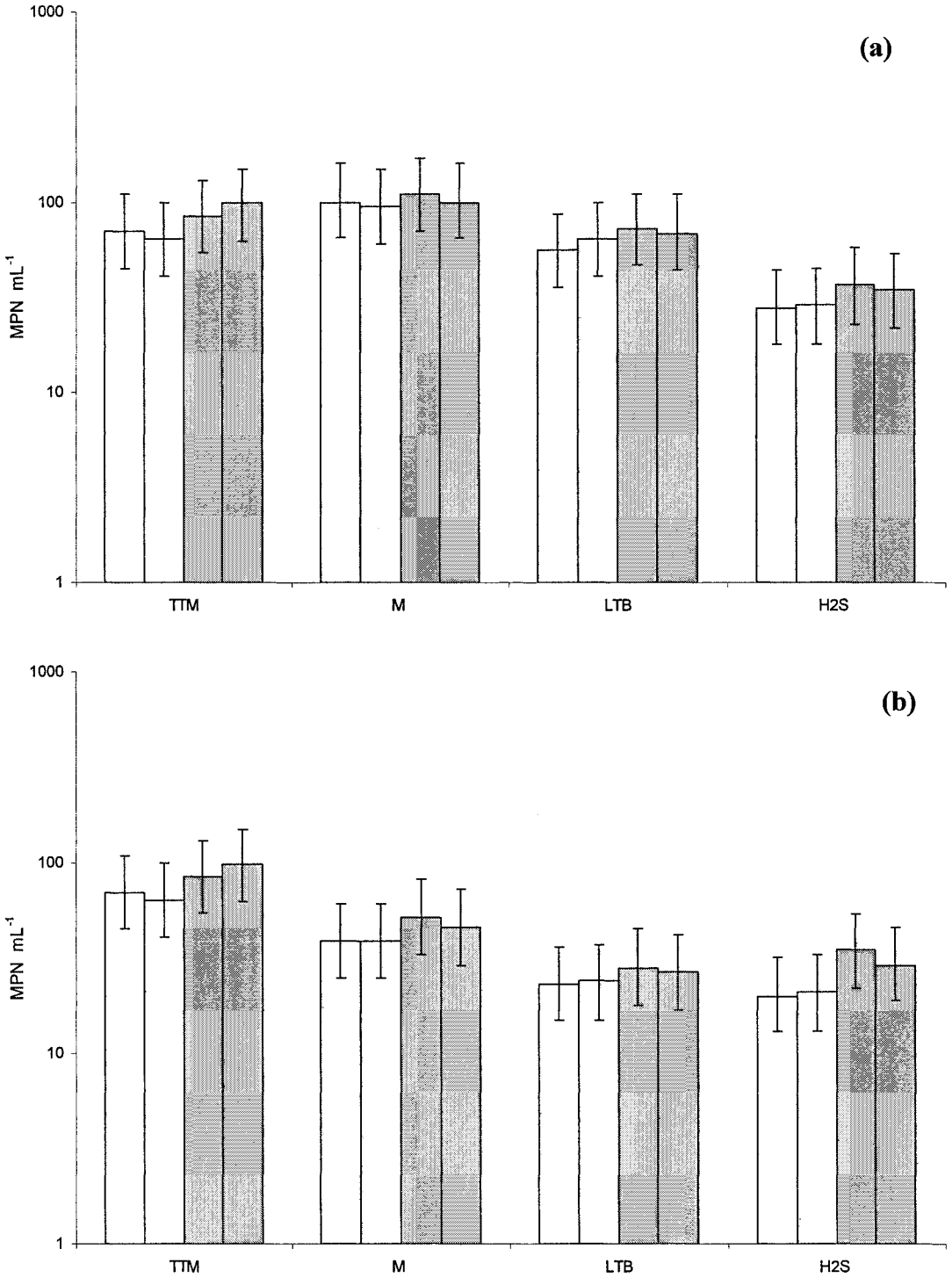


Fig. 7.9 Comparison of novel broth-based media with standard media using sewage contaminated water sample from PGI block-II in India (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on TTM, MacConkey broth (M), lauryl tryptose broth (LTB), and H_2S test medium (H_2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

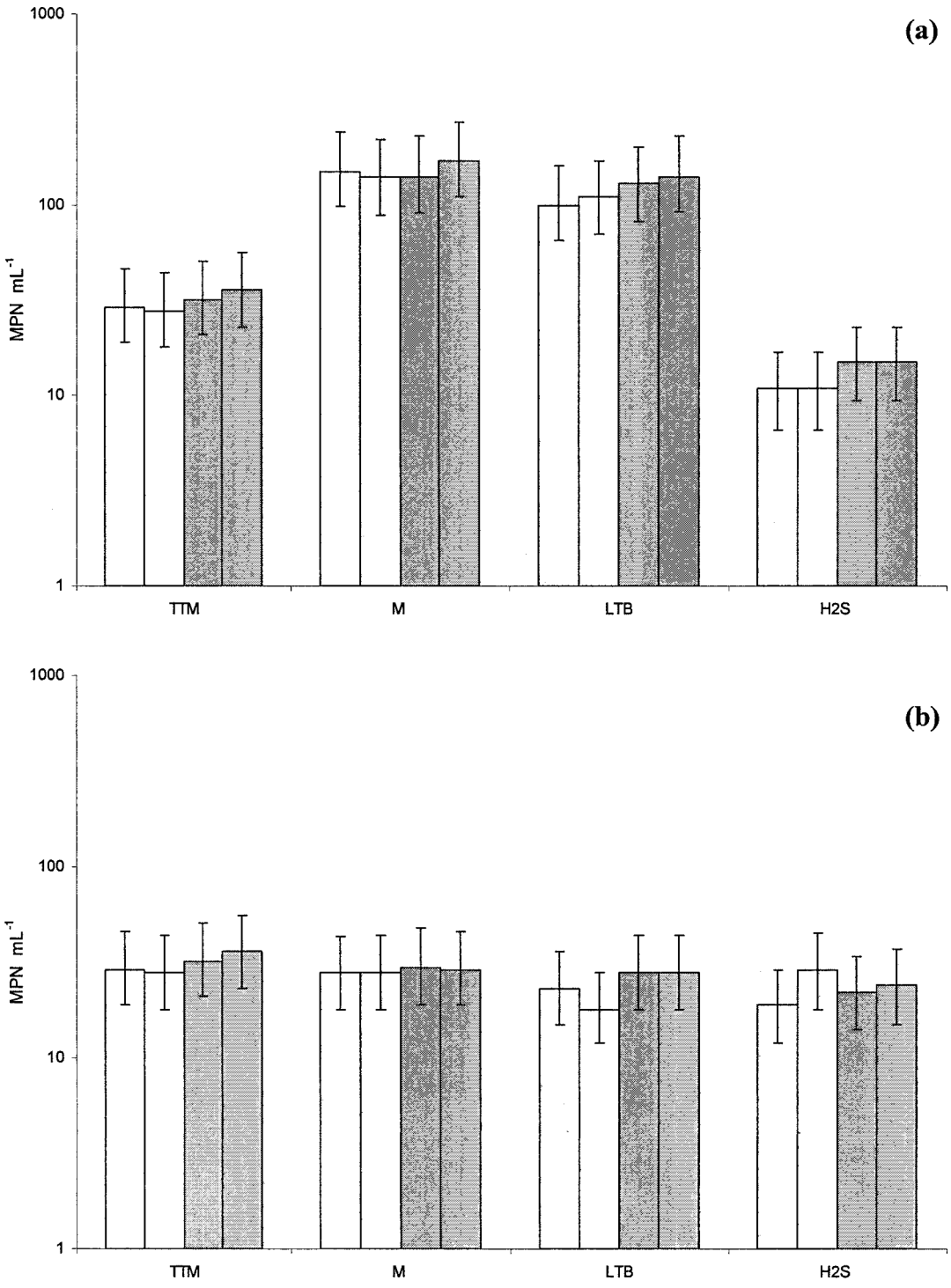


Fig. 7.10 Comparison of novel broth-based media with standard media using sewage contaminated water sample from PGI block-II in India (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on TTM, MacConkey broth (M), lauryl tryptose broth (LTB), and H_2S test medium (H_2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

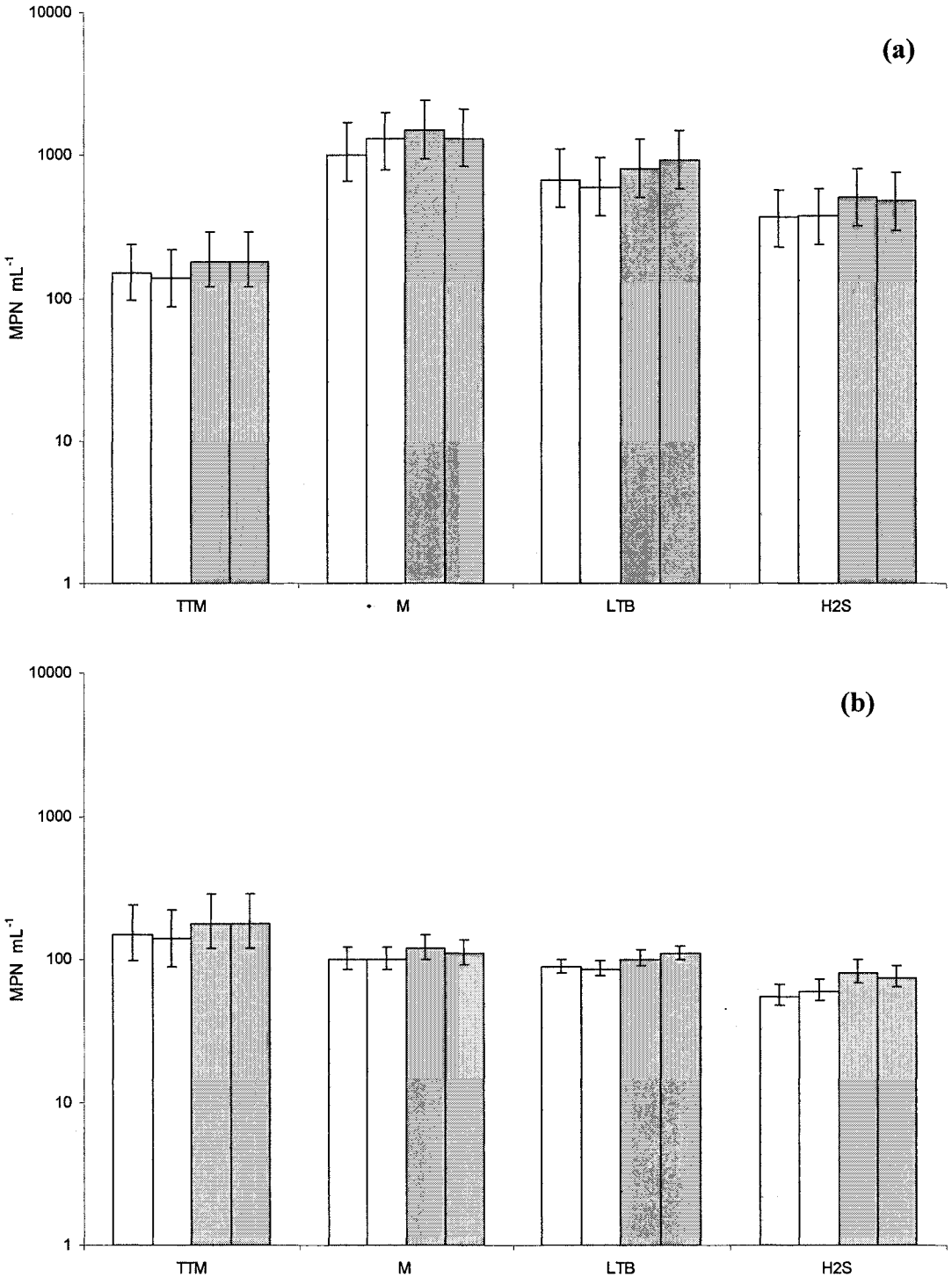


Fig. 7.11 Comparison of novel broth-based media with standard media using sewage contaminated water sample from PGI block-I in India (a) Presumptive MPN value per mL, (b) Confirmed MPN value per mL; under aerobic conditions with growth medium supplemented with 0.05% w/v sodium pyruvate (dark-shaded bars) and unsupplemented growth medium (unshaded bars), enumerated in duplicate on TTM, MacConkey broth (M), lauryl tryptose broth (LTB), and H_2S test medium (H_2S). Error bars represent 95% confidence limits. Note that the vertical axis is log-transformed.

7.3.2 Development of "Coliblack" field medium and its comparison with TTM

Table 7.3 and Plate 7.2 shows results for various concentrations of 8-HQ-GLUC and ferric ammonium sulphate in a single tube format after addition to the basal medium for 24 hour and 48 hour incubation of *E. coli* NCTC8912. The results showed that a combination of 150 or 200 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium sulphate in basal medium were the most effective concentrations to give positive results, i.e. the formation of a black-coloured complex of iron-8-hydroxyquinoline for β-glucuronidase-positive *E. coli* by visualisation of colouration after a 48 hour incubation period. However it was also noted that after autoclaving the mixture of 8-HQ-GLUC and ferric ammonium sulphate in basal medium formed a turbid broth that could hinder the visual examination of positive results and also might be difficult to dry onto filter paper in subsequent developments, as discussed latter.

Table 7.3 Optimising the concentrations of 8-hydroxyquinoline-glucuronide and ferric ammonium sulphate in the basal medium inoculated with *E. coli* NCTC8912. The amount of growth in each test-positive bottle was noted after 24 h and 48 h.

1. - = no growth
2. + = slight growth
3. ++ = moderate growth
4. +++ = high growth
5. ++++ = maximum growth

8-hydroxyquinoline glucuronide (8-HQ-GLUC mg L^{-1})	**Ferric ammonium sulphate (FAS g L^{-1})**	**24 (h)**	**48 (h)**
50	0.25	+	++
100	0.50	++	+++
150	0.75	++	+++
200	1.0	++++	++++
150	1.0	++	++++
100	1.0	+	+++
50	1.0	-	++

Plate 7.2 Photographic combination of different concentrations of 8-hydroxyquinoline-glucuronide and ferric ammonium sulphate (FAS) in the basal medium. These were used for the development of the novel field medium. Numbering from left to right:-

1. 50 mg L^{-1} of 8-HQ-GLUC and 0.25 g L^{-1} of ferric ammonium sulphate.
2. 100 mg L^{-1} of 8-HQ-GLUC and 0.50 g L^{-1} of ferric ammonium sulphate.
3. 150 mg L^{-1} of 8-HQ-GLUC and 0.75 g L^{-1} of ferric ammonium sulphate.

1. 50 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium sulphate.
2. 100 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium sulphate.
3. 150 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium sulphate.
4. 200 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium sulphate

Table 7.4 and Plate 7.3 shows results for different concentrations of 8-HQ-GLUC and ferric ammonium citrate in a single tube format added in basal medium for up to 48-hour incubation period. After 24 hour it was noted that 150 mg L^{-1} of 8-HQ-GLUC + 1.0 g L^{-1} of ferric ammonium citrate, or 200 mg L^{-1} of 8-HQ-GLUC and 0.50 g L^{-1} or 1.0 g L^{-1} of ferric ammonium citrate in the basal medium were the most effective concentrations giving a black-coloured complex while in the case of the results after 48 hour, all the above combinations in addition to 150 mg L^{-1} of 8-HQ-GLUC + 0.375 g L^{-1} of ferric ammonium citrate gave strong positive results. The combination of 150 mg L^{-1} of 8-HQ-GLUC + 1.0 g L^{-1} ferric ammonium citrate in basal medium that gave the highest absorbance (2.6) is shown in Table 7.5. The different combination of 8-HQ-GLUC + ferric ammonium citrate are shown in Plate 7.4. The combination of both ingredients was selected keeping in mind the possible inhibitory effect of high levels of 8-HQ-GLUC. Thus in all subsequent experiments a concentration of 150 mg L^{-1} for 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate was added into the basal medium.

Table 7.4 Optimising the concentrations of 8-hydroxyquinoline-glucuronide and ferric ammonium citrate in the basal medium inoculated with *E. coli* NCTC8912. The amount of growth in each test-positive bottle was noted after 24h and 48 h.

- = no growth
+ = slight growth
++ = moderate growth
+++ = high growth
++++ = maximum growth

8-hydroxyquinoline glucuronide (8-HQ-GLUC mg L^{-1})	**Ferric ammonium citrate (FAC g L^{-1})**	**24 (h)**	**48 (h)**
50	0.125	+	++
100	0.25	+++	+++
150	0.375	+++	++++
200	0.50	++++	++++
200	1.0	++++	++++
150	1.0	++++	++++
100	1.0	+++	+++
50	1.0	++	++

Table 7.5 Absorbance (550 nm) given by each test-positive combination of 8-hydroxyquinoline glucuronide and ferric ammonium citrate in the basal medium.

8-hydroxyquinoline glucuronide (8-HQ-GLUC mg L^{-1})	**ferric ammonium citrate (FAC g L^{-1})**	**absorbance obtained after 24 hours**
100	0.25	1.8
100	0.50	1.7
100	0.10	1.5
150	0.375	1.0
150	0.75	2.5
150	1.0	2.6
150	1.5	2.0
200	1.0	2.0

Plate 7.3 Photographic view of combination of different concentrations of 8 hydroxyquinoline-glucuronide and ferric ammonium citrate in the basal medium. These were used for the development of novel field medium. Numbering from left to right:-

1. 50 mg L^{-1} of 8-HQ-GLUC and 0.125 g L^{-1} of ferric ammonium citrate.
2. 100 mg L^{-1} of 8-HQ-GLUC and 0.250 g L^{-1} of ferric ammonium citrate.
3. 150 mg L^{-1} of 8-HQ-GLUC and 0.375 g L^{-1} of ferric ammonium citrate.
4. 200 mg L^{-1} of 8-HQ-GLUC and 0.50 g L^{-1} of ferric ammonium citrate.
5. 200 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate.

1. 50 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate.
2. 100 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate.
3. 150 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate.

Plate 7.4 Photographic view of the test-positive (black colouration) bottles used for measuring absorbance for different concentrations of 8 hydroxyquinoline-glucuronide and ferric ammonium citrate in the basal medium. Numbering from left to right:-

1. 100 mg L^{-1} of 8-HQ-GLUC and 0.25 g L^{-1} of ferric ammonium citrate.
2. 100 mg L^{-1} of 8-HQ-GLUC and 0.50 g L^{-1} of ferric ammonium citrate.
3. 100 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate.
4. 150 mg L^{-1} of 8-HQ-GLUC and 0.375 g L^{-1} of ferric ammonium citrate.
5. 150 mg L^{-1} of 8-HQ-GLUC and 0.75 g L^{-1} of ferric ammonium citrate.
6. 150 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate.
7. 150 mg L^{-1} of 8-HQ-GLUC and 1.5 g L^{-1} of ferric ammonium citrate.
8. 200 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate.

Figure 7.12 a-j displays results for presumptive and confirmed MPN values obtained for *E. coli* isolated from water samples by enumeration using basal medium with enzyme-substrates MU-GLUC or 8-HQ-GLUC with incubation at temperatures of 25°C and 37°C. The MPN value per mL was similar whether using MU-GLUC or 8-HQ-GLUC as a substrate for testing β-glucuronidase-positive bacteria. The presumptive and the confirmed counts at incubation temperatures of 25°C were slightly higher for both substrates in almost all 10 water samples compared with the corresponding count at 37°C, indicating that lower temperatures did not give false-positive results and recovered a higher overall count of *E. coli*. There was also a good agreement between presumptive and confirmed MPN values.

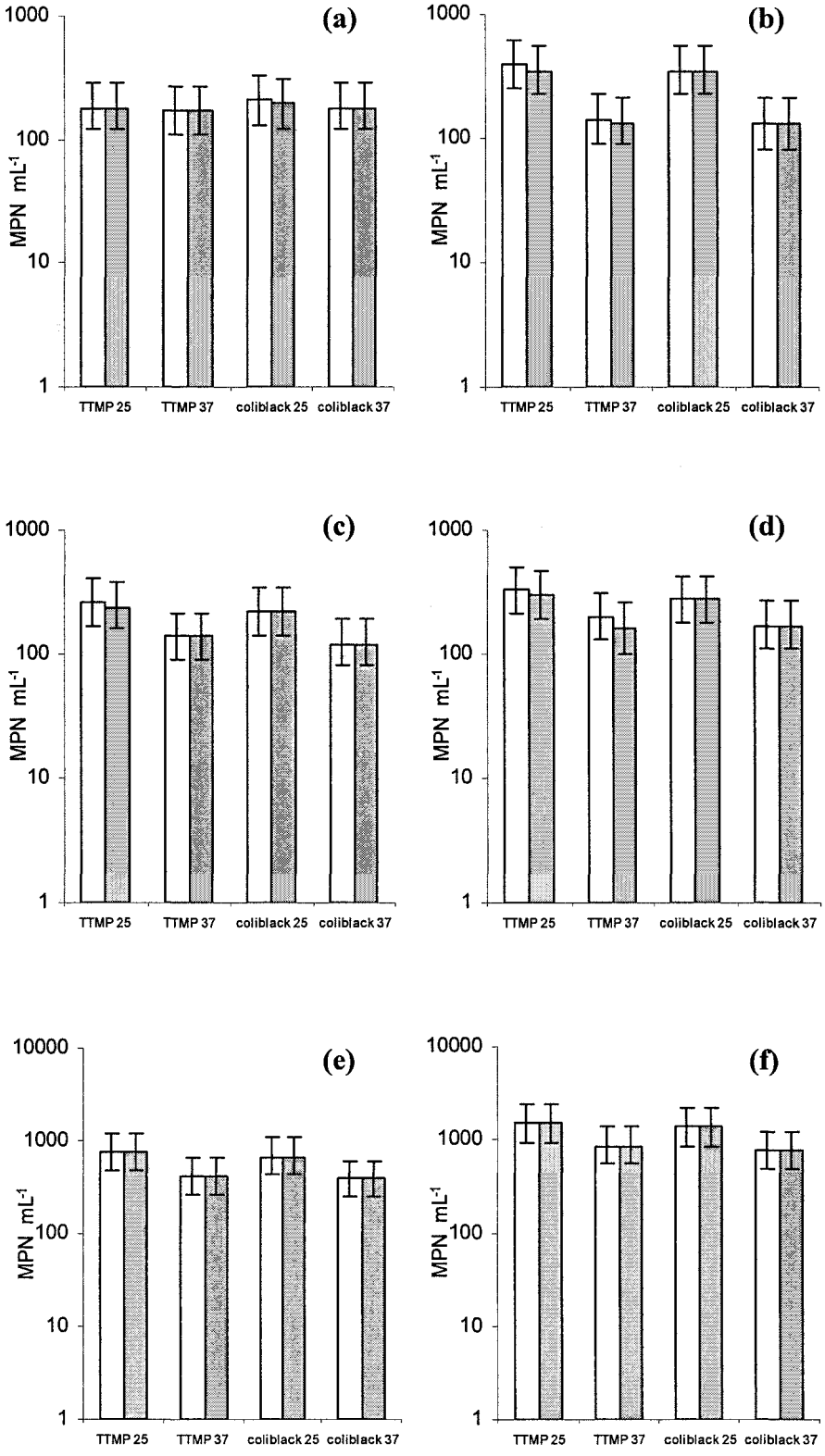


Fig. 7.12 a-f Comparison of TTMP with coliblack in broth-based medium in MPN multiwell assay at incubation temperatures of 25°C and 37°C (a) Doraha river sample, (b) Khamanon river sample, (c) Kajauli river sample, (d) PGI Block-I sewage sample, (e) PGI Block-II sewage sample, (f) PGI Block-I sewage sample The MPN per mL are shown for the presumptive values (unshaded bar) and confirmed values (dark-shaded bars). Note that the vertical axis is log-transformed (key to the graph overleaf).

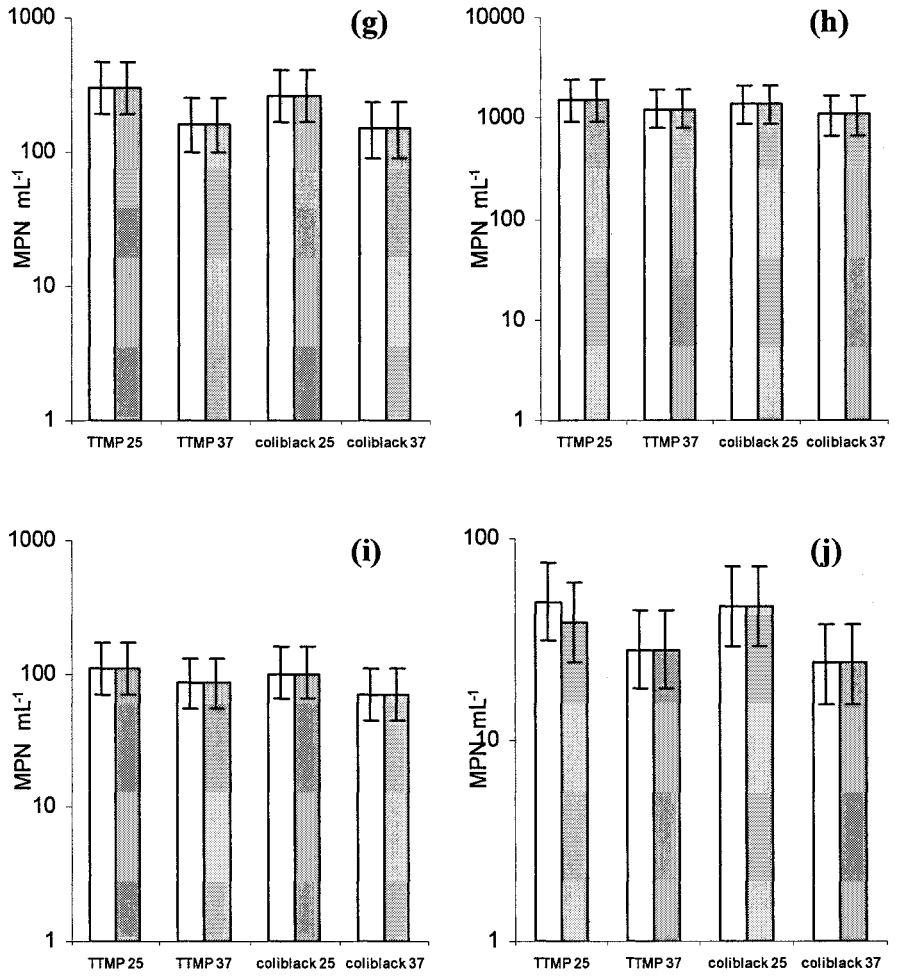


Fig. 7.12 g-j Comparison of TTMP with coliblack in broth-based medium in MPN multiwell assay at incubation temperatures of 25°C and 37°C (g) PGI Block-II sewage sample, (h) Doraha river sample, (i) Khamanon river sample, and (j) Kajauli river sample. The MPN per mL are shown for the presumptive values (unshaded bar) and confirmed values (dark-shaded bars). Note that the vertical axis is log-transformed.

TTMP 25- basal medium + X-GLUC + incubated at 25°C.
TTMP 37- basal medium + X-GLUC + incubated at 37°C.
Coliblack 25- basal medium + X-GLUC + incubated at 25°C.
Coliblack 37- basal medium + 8-HQ-GLUC + ferric salt incubated at 37°C.

7.3.3 Comparison of "Coliblack-12" and "H_2S-12" with a plate counting method using Chromocult agar

Fig. 7.13a shows presumptive MPN values obtained after screening 50 water samples, in XY-scatter graph format, with each data point representing log-transformed paired MPN values obtained after enumeration in the four dilution bottle format in triplicate with H_2S-12 plotted on the X-axis and Coliblack-12 plotted on the Y-axis as per Report 71 (Anon., 2002; see also Chapter 5). The theoretical line of equivalence is represented by the dotted line. The results show that out of 50 samples, four sample results were the same for both methods and lay on line of equivalence, 1 lay towards the X-axis and 45 lay towards the Y-axis. Thus 98% of the MPN log-paired values were higher by Coliblack-12 when compared to H_2S-12. Using the Wilcoxon signed rank test (T) on this presumptive data there is a significant difference between the two test methods for the 45 ranked samples, as T < critical value (C), i.e. T=49, C=292 (N= 45) at a *P* value of 0.01 (Kirkwood *et al.*, 2003). Plate 7.5 shows photographic comparison between Coliblack-12 and H_2S-12

The confirmed results of presumptive values from Fig. 7.13a plotted as log-paired MPN values obtained for *Escherichia coli* are shown in Fig. 7.13b, with overall eight samples from sources giving the same value by both assays; the remaining 42 paired results were higher for Coliblack-12, thus giving 100% higher values than for H_2S-12. This set of results indicates that H_2S-12 consistently underestimated the MPN of the tested samples for *E. coli*, when compared with Coliblack-12.

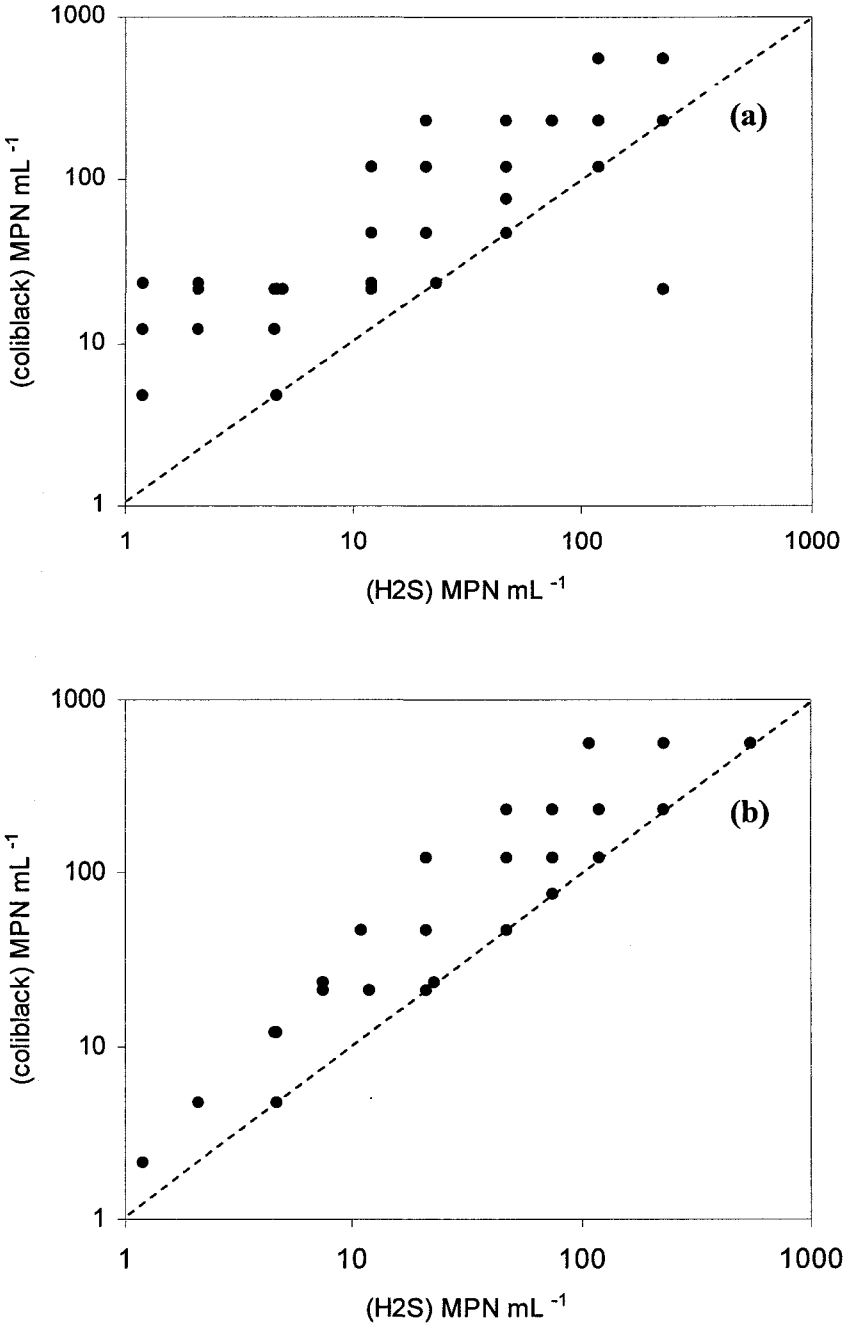


Fig. 7.13 Comparison of "Coliblack-12" with "H_2S-12" using broth-based format (a) Presumptive value for MPN mL^{-1}, (b) Confirmed value for MPN mL^{-1} with each data point representing log-transformed paired MPN values obtained for triplicate bottles using MPN method for H_2S-12 plotted on the X-axis and coliblack-12 plotted on the Y-axis. The theoretical line of equivalence is represented by dotted line. Note that the vertical axis is log-transformed.

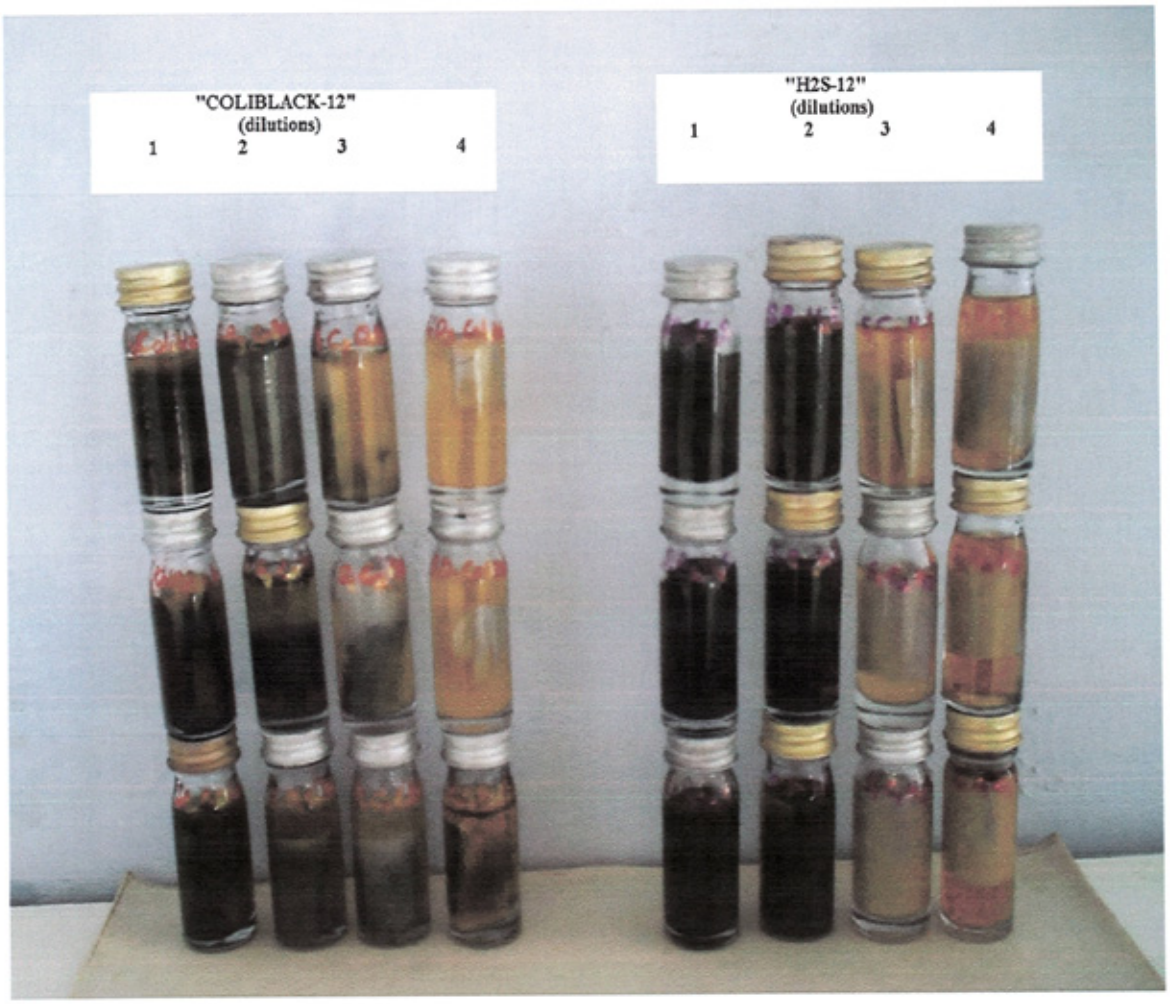


Plate 7.5 Photographic comparison between "Coliblack-12" and "H_2S-12" bottles in triplicate with 3, 3, 3, 1 bottles giving test-positive results using "Coliblack-12" as the field medium and 3, 3, 0, 0 bottles giving test-positive results using "H_2S-12"as the field medium.

Presumptive results in Fig. 7.14a are shown in XY-scatter graph format, with H_2S-12 (MPN) plotted on the X-axis and the MF method using Chromocult agar plotted on the Y-axis. Analysis of log-paired values shows that out of 50 test sources, all but one gave a higher result for the MPN method using H_2S-12, compared to MF method using Chromocult agar H_2S-12 (49 values higher). Thus the results indicate H_2S-12 to be recovering fewer numbers of bacteria than Chromocult agar in most samples.

The presumptive counts from Fig. 7.14a after confirmation are plotted as log-paired MPN values for *Escherichia coli* in Fig. 7.14b, with only three confirmed MPN values higher by H_2S-12, while the rest of the forty seven values confirmed higher counts by Chromocult agar. Thus again H_2S-12 proved to underestimate the true count, which was consistently lower in the medium compared to Chromocult agar.

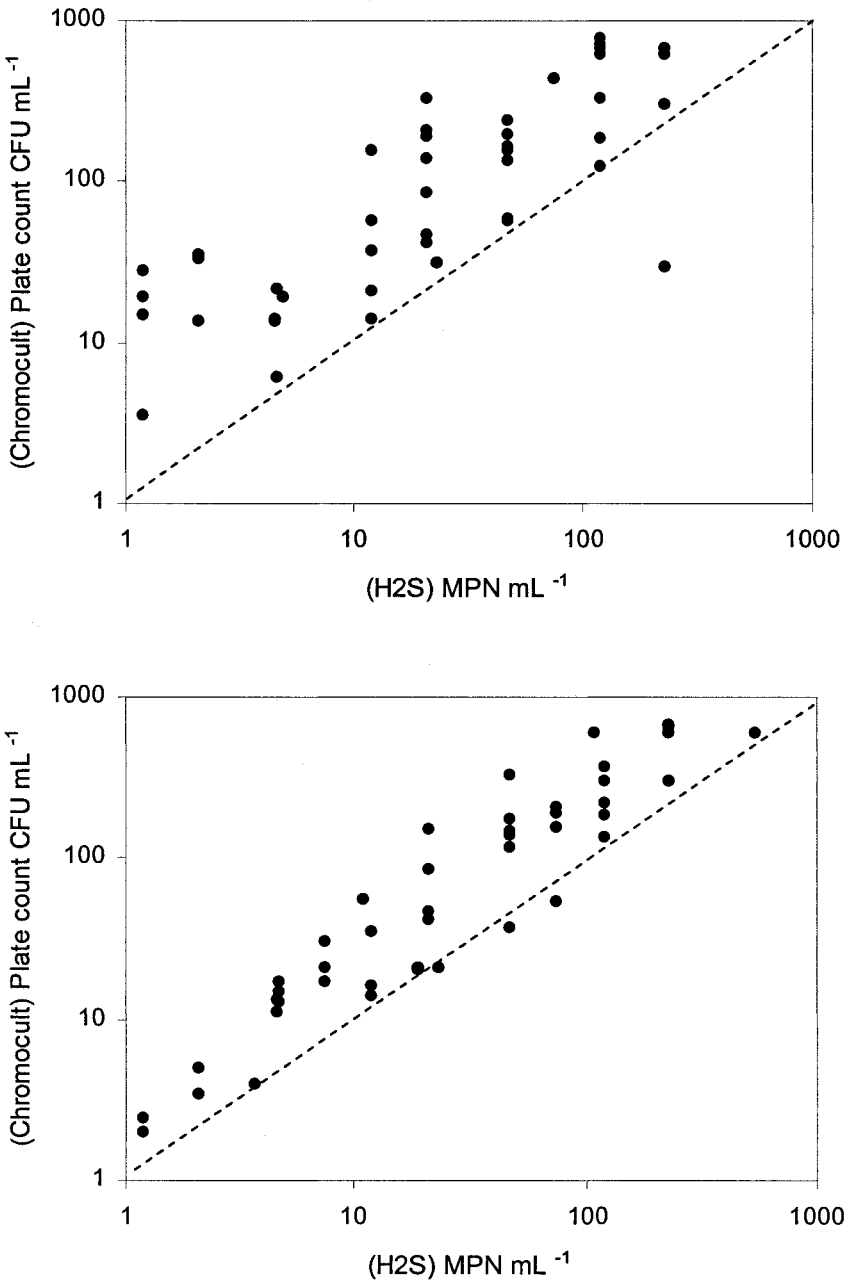


Fig. 7.14 Comparison of "H_2S -12" with a plate counting method using Chromocult agar (a) Presumptive value for MPN mL^{-1}, (b) Confirmed value for MPN mL^{-1} with each data point representing log-transformed paired values obtained for triplicate using plate count method for Chromocult agar plotted on the X-axis and MPN method for H_2S-12 plotted on the Y-axis. The theoretical line of equivalence is represented by dotted line. Note that the vertical axis is log-transformed.

Figure 7.15 displays results obtained when comparing presumptive test-positive H_2S-12 (X-axis) MPN per mL with confirmed H_2S-12 test growth-positive confirmed (Y-axis) MPN per mL. The analysis of log-paired MPN values show, 12 tests out of 50 gave similar values, 5 tests gave higher values in case of presumptive count and 33 tests gave higher confirmed values, i.e. based on growth-positive data. The Wilcoxon signed rank test showed a significant difference between confirmed (growth-positive) H_2S-12 test and presumptive (test-positive) H_2S-12 tests MPN values, as T < critical value (C), i.e. T=141, C=373 (N= 38) at a *P* value of 0.01 (Kirkwood *et al.*, 2003).

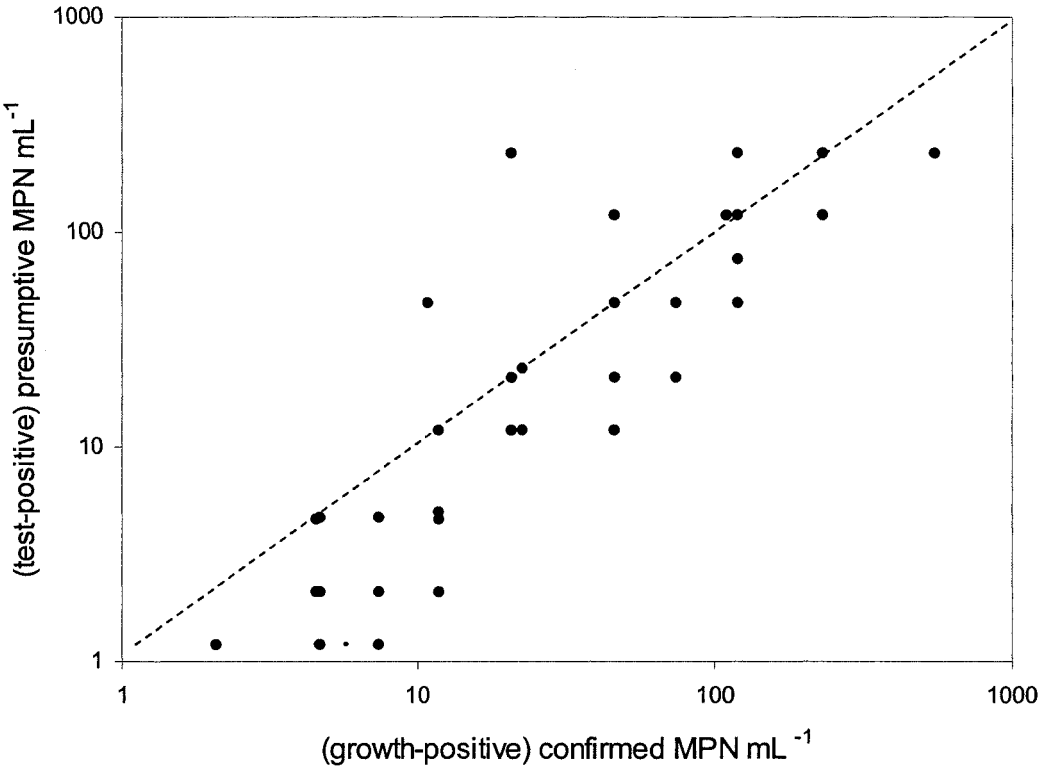


Fig. 7.15 Comparison of growth-positive and test-positive MPN values for "H_2S- 12" medium (a) presumptive (test-positive) value for MPN mL^{-1}, (b) confirmed (growth-positive) value for MPN mL^{-1}, with each data point representing log-transformed paired values obtained for triplicate bottles each for four dilutions using MPN method for confirmed growth-positive results plotted on the X-axis and presumptive test-positive results plotted on the Y-axis. The theoretical line of equivalence is represented by dotted line. Note that the vertical axis is log-transformed.

Fig. 7.16a provides comparative results for Chromocult agar-based MF (X-axis), or Coliblack-12 based MPN techniques (Y-axis). The analysis of log-paired MPN values show that out of 50 sources, one test sample gave similar count by both methods, 23 gave higher count for MF method using Chromocult agar while 26 gave higher MPN values using Coliblack-12. The Wilcoxon signed rank test (T) concluded that there was no significant difference between both media for 49 samples analyzed, as T < critical value (C), i.e. T=475, C=415 (N= 49) for two-sided *P* value of 0.05.

The confirmed data for *Escherichia coli* (Fig. 7.16b) shows 15 log-paired results out of 50 confirming higher count by Chromocult agar and 35 out of 50 confirming for Coliblack-12. The Wilcoxon signed rank test (T) interpreted that there was no significant difference in performance within both enumeration media as, T< critical value (C), i.e. T=490, C=434 (N= 50) at a *P* value of 0.05.

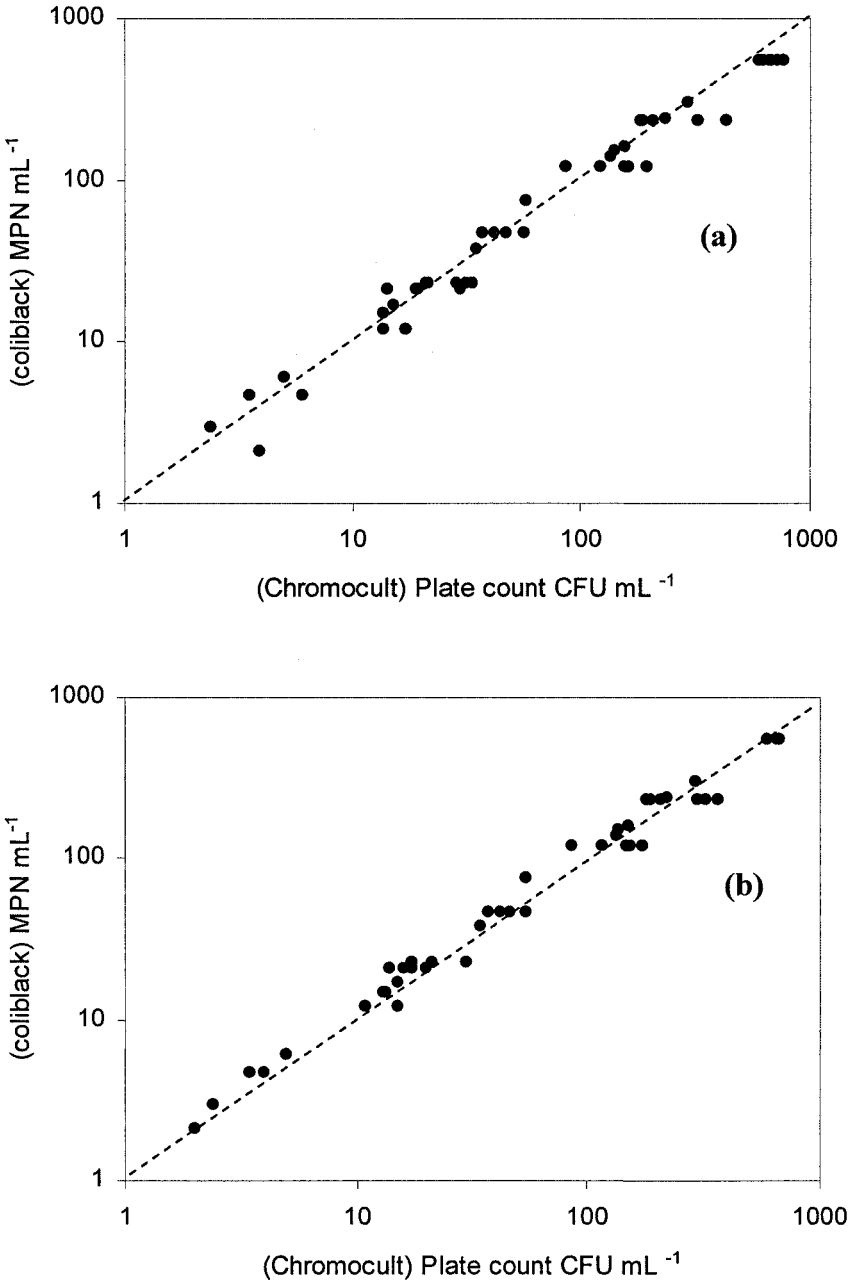


Fig. 7.16 Comparison of "Coliblack-12" with a plate counting method using Chromocult agar (a) Presumptive value, (b) Confirmed value, with each data point representing log-transformed paired values obtained for triplicate using plate count method for Chromocult agar plotted on the X-axis and MPN method for Coliblack-12 plotted on the Y-axis. The theoretical line of equivalence is represented by dotted line. Note that the vertical axis is log-transformed.

7.3.4 Field evaluation of "Coliblack-4"

The evaluation feedback from the field tests of Coliblack-4 medium given to unskilled subjects are summarised in Table 7.6. The 12 subjects used the instructional sheet described earlier which they commented to be easy to understand as 100% of the closed types of questions were answered (yes). The open type of questions gave their opinions and comments after performing the test, showing positive feedback from their perspective as they indicated the test to be easy to perform and to follow using the instructional sheet. The comments about the open type of questions were positive. One of the specific comments about the instructional sheet was, for example one of the subjects (SD) was confused by the word "mix" written in the first line of the instructional sheet as she thought needed something (e.g. a stirrer) with which to mix the medium. This could be remedied by replacing the word "turn bottles slowly upside down and back again for six times". All of the 12 subjects were fully able to understand the language used to perform the steps written in the instructional sheet. The drinking water samples of all of the 12 subjects were found to be contaminated with faecal bacteria based on the number of positive bottles in each test which varied from slight contamination to highly contaminated drinking water. Overall, despite the small size of the pilot scale study, the results are encouraging and indicate that this approach may be worthy of further development.

Table 7.6 Field evaluation of "Coliblack 4" with the help of an instructional sheet provided to 12 subjects for testing drinking water quality and to obtain feedback from them in the form of comments/opinions about the test.

Subject Water source: Tap or Handpump	Number of positive bottles	Interpretation	Time period for positive result (hour)	Percentage of positive feedback in closed type questions	Special comments (open questions)
1. P T (tap water)	1	Slightly contaminated	24	100%	water unfit for drinking.
2. R M (tap water)	2	Moderately contaminated	48	100%	water contaminated for drinking.
3. K S (handpump)	3	Highly contaminated	48	100%	water is highly contaminated for drinking and 100%.
4. J S (handpump)	1	Slightly contaminated	48	100%	black precipitation in one bottle therfore water unhygienic for drinking.
5. K S (tap water)	1	Slightly contaminated	24	100%	black precipitation in one bottle so slight contaminated drinking water.
6. S D (tap water)	1	Slightly contaminated	24	100%	one bottle turned black so water is contaminated; word "mix" in instructional sheet confusing.
7. P K (tap water)	2	Moderately contaminated	24	100%	water is dirty to drink
8. R S (tap water)	2	Moderately contaminated	24	100%	black precipitation in two bottles, test quite handy.
9. P B (tap water)	1	Slightly contaminated	48	100%	water slightly contaminated.
10. S S (tap water)	1	Slightly contaminated	24	100%	slight contamination.
11. N P (handpump)	1	Slightly contaminated	24	100%	drinking and bathing water contaminated.
12. M C (handpump)	3	Highly contaminated	48	100%	water is highly unfit to drink.

7.4 Discussion

The importance of fluorogenic and chromogenic glycosidase substrates as identification markers have been known for many years for the detection of specific enzymes and the identification of bacteria such as *Escherichia coli* in bacterial diagnostics (Manafi *et al.*, 1991; Butterworth *et al.*, 2004). They are also used for quantitative determination of total coliforms and *E. coli* in marine waters (Geissler *et al.*, 1998; 2000), presence or absence of total coliforms using Colisure (McFeters *et al.*, 1995), or Colilert systems in drinking waters (Edberg and Edberg, 1988; Edberg *et al.*, 1988; Manafi, 1998), for marine and freshwater recreational bathing water samples (Budnick *et al.*, 1996, IDEXX, Westbrook, Maine), for determination of *E. coli* in drugs (Huang *et al.,* 1994), in foods of animal origin (Bredie and de Boer, 1992) and for isolation of diarrheaogenic *E. coli* (Muto *et al*; 1991). In formulating new broth-based or agar-based growth media with glycosidase substrates, the presence of the enzyme β-glucuronidase (GUD) is generally regarded as a suitable indicator for *E. coli* (Berg *et al.,* 1988; Chapman *et al.*, 1991; Rice *et al.*, 1991; Larinkari and Rautio, 1995; Brenner *et al.*, 1996; Manafi, 1996; Alonso *et al.,* 1999; Restaino *et al.*, 1999; Butterworth *et al.*, 2004) as 94-96% of *E. coli* strains are β-glucuronidase-positive (Hartman, 1989; Manafi, 2000), for example James and Yeoman (1988) introduced the glycosidase substrate 8-hydroxyquinoline-β-glucuronide that gave positive results when *E. coli* was present (James *et al.*, 1996; James *et al.*, 2000b). The inclusion of such synthetic enzyme substrates into primary isolation media had led to rapid detection, improved accuracy, specificity and sensitivity of detection of the target organism thereby decreasing the time consumed in performing confirmatory tests (Manafi, 1998; 2000).

In the present study two β-glucuronide substrates, i.e. 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-GLUC) and 4-methylumbelliferone-β-glucuronide (MU-GLUC) were compared by their incorporation into a basal medium for the evaluation of a broth-based medium. The presumptive results obtained for water samples in Figure 7.1a, 7.3a-7.11a and confirmed results for *Escherichia coli* in Figure 7.1b, 7.3b-7.11b gave similar count using the novel broth based medium irrespective of which enzyme-substrate was used. Moreover the maximum number of presumptive tests confirmed as *Escherichia coli* using such novel broth medium while those having lactose fermentation as their indicator gave a substantial number of false-positives. In contrast, whereas maximum numbers of false-negatives were obtained for the H_2S test medium. Thus it is clear from these results that they contradict the WHO report (WHO, 2002b) where the H_2S test medium was regarded as a medium of choice for the detection of faecal coliforms since the present study shows that this medium consistently underestimates the number of *Escherichia coli* present in water samples.

James *et al.* (2000a) formulated a novel chromogenic substrate termed as *p*-naphtholbenzein-β-galactopyranoside (PNB-GAL) incorporated in Columbia agar that on hydrolysis gave *p*-naphtholbenzein, forming pink-coloured colonies; the effectiveness of this substrate was compared against other two substrates i.e. cyclohexenoesculetin-β-galactoside (James *et al.*, 1996; 1997) that hydrolyzed to form a metal-chelate with iron, producing a black coloured colonies (CHE-GAL), and X-Gal hydrolyzing to produce blue colonies for bacteria such as total coliforms or *E. coli* possessing the enzyme β-galactosidase. Butterworth *et al.*, (2004) evaluated two chromogenic substrates incorporated in Columbia agar, namely 3', 4'-dihydroxyflavone-4'-β-ribofuranoside (DHF-riboside) that is hydrolysed by β-ribosidase-enzyme possessing bacteria such as *E. coli* to release 3', 4'-

dihydroxyflavone that in presence of ferric salt forms an insoluble black chelate-like complex, 5-bromo-4-chloro-3-indolyl-β-ribofuranoside (X-riboside) that hydrolyses to form an insoluble blue-green complex in presence of oxygen and compared with a fluorogenic substrate 4-methylumbelliferone-β-ribofuranoside (4MU-riboside) for assessing β-ribosidase activity for differentiation and in the identification of pathogenic Gram-negative bacteria possessing this enzyme. James and Yeoman (1988) investigated the use of the substrate, 8-hydroxyquinoline-β-glucuronide (400 mg L^{-1}) and a ferric salt, i.e. ferric ammonium citrate (1.0 g L^{-1}) in basal medium for testing the activity within the family Enterobacteriaceae (Reinders *et al.*, 2000) and concluded that its inclusion in agar-based media formed an intense black pigmentation localised around the colony mass because of the formation of an iron-8-hydroxyquinoline complex which yielded positive results only for *Escherichia coli* (James *et al.*, 2000b). Similarly Reinders *et al.* (2000) formulated a medium for presumptive identification and isolation of Shiga toxin-producing *E. coli* O157 constituting 8-hydroxyquinoline-β-glucuronide (200 mg L^{-1}) and ferric ammonium sulphate (1.0 g L^{-1}) included in Sorbitol MacConkey agar. The preliminary results for optimisation of substrate and iron concentration in the present study observed the production of a turbid solution after autoclaving a mixture of 8-hydroxyquinoline-β-glucuronide and ferric ammonium sulphate (Table 7.3) while a mixture of 8-hydroxyquinoline-β-glucuronide and ferric ammonium citrate produced no such turbidity that was thus selected as the iron source in the basal medium in subsequent experiments (Table 7.4 and 7.5).

Reinders *et al.* (2000) noted that the higher concentrations of 8-hydroxyquinoline could inhibit the growth of *E. coli* by making the iron source unavailable for growth in nutrient agar. Similarly James and Yeoman (1987; 1988) suggested the possible

inhibition of bacteria due to release of 8-hydroxyquinoline that could make iron unavailable for growth. The anti-microbial property of 8-hydroxyquinoline has been studied in the past and is found to be most prominent in Gram-positive bacteria such as staphylococci (Albert *et al.*, 1953) and also in some Gram-negative bacteria (Rubbo *et al.*, 1950) where high concentrations make iron source unavailable by forming complexes (Reinders *et al.* 2000). In the present study experiments were carried out to examine the most effective concentration of 8-HQ-GLUC using lower concentrations because of this possible inhibitory nature of 8-hydroxyquinoline along with ferric ammonium citrate, thus 150 mg L^{-1} of 8-HQ-GLUC and 1.0 g L^{-1} of ferric ammonium citrate was selected on the basis of growth of *E. coli*, colour change and absorbance as the most effective concentrations.

The comparisons between two substrates, i.e. MU-GLUC and 8-HQ-GLUC introduced in the basal medium recovered similar *E. coli* numbers from water samples (Fig. 7.12). These results are somewhat contradictory to that of James and Yeoman (1988) where differences in MPN values between both substrates was noted that may be explained by different basal media used in two studies, with the present research incorporating sodium pyruvate that have been regarded as a resuscitative agent and tergitol-7 that is a minimum inhibitory selective agent for the growth of injured bacteria (LeChevallier *et al.*, 1983; McFeters *et al.*, 1986; Calabrese and Bissonnette, 1990a; Sartory, 1995; Clesceri *et al.,* 1998). Similarly Reinders *et al.* (2000) noted a lesser difference in count using the substrates MU-GLUC and 8-HQ-GLUC incorporated in selective Sorbitol MacConkey agar that they explained might be due to the presence of bile salts in the selective medium used. Fujisawa and Moris (1997) also noted an increase of β-glucuronidase activity in presence of bile salts.

In the present study the presumptive and the confirmed count at the incubation temperatures of 25°C were higher for both substrates in all 10 water samples from the corresponding count at 37°C, indicating that in final evaluation of the field-based medium, incubation at lower temperatures than present in the conventional incubator would not necessarily adversely effect the overall recovery rate of *E. coli*. Moreover lower concentrations of 8-hydroxyquinoline-β-glucuronide were used in conjugation with higher concentration of ferric salt that proved to be less inhibitory because of the lesser chances of formation of toxic 1:1 complex (Reinders *et al.*, 2000) that were supported by the similar results obtained for both substrates such as 8-HQ-GLUC and MU-GLUC incorporated in basal medium (Fig. 7.12).

In the present study comparisons between H_2S-12 and Coliblack-12 (four dilution bottle format in triplicate) and Chromocult agar showed significant differences between H_2S-12 and Coliblack-12 and also between Chromocult agar and H_2S-12 both in presumptive and confirmed results. Coliblack-12 recovered highest number of *E. coli* and gave minimum false-negatives or false-positives followed by Chromocult agar, while H_2S-12 gave the highest number of false-negatives in the presumptive MPN value (Fig. 7.13a-b, 7.14 a-b, 7.15 and 7.16a-b). Thus the results contradict the concept that the hydrogen-sulphide test can be used as an alternative indicator of faecal contamination in water suggested by Manja *et al.* (1982) and recommended by WHO (2002b) as a simple test for testing the bacteriological quality of water (Kromoredjo and Fujioka, 1991; Castillo *et al.,* 1994; Martins *et al.*, 1997; Pillai *et al.*, 1997; Rijal *et al.*, 2000; Manja *et al.*, 2001).

Coliblack was incorporated with 8-hydroxyquinoline-glucuronide along with ammonium ferric citrate in the basal medium that could also be used for the enumeration of an anaerobic incubated samples as 8-HQ-GLUC and iron source form a complex independently of the presence or absence of oxygen which is normally needed for the formation of colour in other chromogenic substrates e.g. X-GLUC (Manafi *et al.*, 1991; James *et al.*, 1996; 2000a; Manafi, 2000). Coliblack-4 is quantitative as it is a four bottle test thus giving a positive result and a MPN value. The diagnostic agent in Coliblack-4 can be used in agar as it does not diffuse, for example in the case of Uricult-Trio agar used for rapid detection of *E. coli* in urine samples (Dalet and Sergosia, 1995; Larinkari and Rautio, 1995), or for the detection of STEC-positive, β-glucuronidase-negative pathogenic *E. coli* O157 strains by incorporation into Sorbitol MacConkey agar (Reinders *et al.*, 2000). Furthermore, this medium needs no laboratory support and provides rapid and specific results without the need for confirmatory steps. Coliblack-4 may help the revival and growth of injured bacteria because of incorporation of resuscitative agent (sodium pyruvate).

The Coliblack-4 test medium when evaluated under field conditions, i.e. at the household level in rural locations suggested the positive operation of the test with the help of the instructional sheet. The feedback from 12 unskilled subjects who were unaware of the detailed microbiological aspects of the test gave almost 100% positive feedback which is evident from their comments, for example the test was easy to perform and the sheet was easy to follow. Furthermore, the drinking water of all 12 subjects was contaminated with faecal bacteria and thus the test created an awareness in those people as to how much they were at the risk to water-borne diseases caused by the faecal bacteria. These people were also further informed about easy to perform methods of disinfection such as storage of water in brass or copper

vessels for 48 h (Chapter 3) or exposing clear transparent plastic bottles to sunlight for solar photo oxidation (Reed, 2004; Khaengraeng and Reed, 2005) for a point-of-use disinfection method (Crump *et al.*, 2004) that have been discussed in detail in Chapters 3 and 4.

Chapter 8

Conclusions and Future Research

8.1 Overview

Water has a supreme importance in sustaining all forms of life (Reed *et al.*, 2005). A number of procedures have been imposed to provide safe drinking water (WHO, 2004a). These procedures include guidelines for acceptable microbiological quality in order to minimise cases of water-borne diseases. The guidelines lay emphasis on detecting and enumerating faecal contaminants of water, primarily the faecal indicator bacteria (Anon., 2002; WHO, 2004b). Traditional culture-based techniques such as the most probable number (MPN) or membrane filtration (MF) procedures used to isolate and enumerate bacteria such as faecal coliforms or faecal streptococci in environmental samples are based on estimating numbers under standard aerobic conditions, which is effective in growing healthy cells while injured bacteria may be unable to grow under such conditions (Rompre *et al.*, 2002; Bjergbaek and Roslev, 2005). Furthermore, the techniques involved in the enumeration of faecal indicators are often based on traditional concepts such as the production of acid and gas from lactose, which is frequently incorporated into growth media used for the isolation of faecal coliforms, e.g. MacConkey agar (Chilvers, 2001), mFC agar (Clesceri *et al.*, 1998), or mLSA agar (Anon., 2002). However these selective media used for such enumeration may be ineffective in growing sub-lethally injured bacteria (McFeters *et al.*, 1986; Sartory, 1995; Anon., 2002; Khaengraeng and Reed, 2005). The presence of selective agents in growth media can be the source of secondary stress for injured cells, as discussed in Chapters 3 and 5.

The aims of the present study were to investigate the effects of various stressors (water kept in the brass vessel or exposure to sunlight, high temperatures, free chlorine, low pH or starvation) in inactivating faecal indicator bacteria in water enumerated under aerobic and conditions designed to neutralise reactive oxygen

species (ROS) and to test currently available methods for the resuscitation of injured faecal indicator bacteria, in comparison with standard US and UK methods and with methods based on ROS-neutralisation (Chapter 1, page 1-39). The broad findings were that sub-lethal injury caused to faecal indicator bacteria by several individual stressful factors present in the environment or induced in the laboratory (Chapters 3 and 4) may also result in oxygen sensitivity in the cells during enumeration. It was noted that ROS-neutralised growth conditions can be effective in enhancing the colony count of sub-lethally injured faecal indicator bacteria, whether they are enumerated using either a non-selective medium, a selective medium (Chapter 3 and 4), a resuscitative medium (Chapter 5), or a chromogenic medium (Chapter 6). In this respect the original notion of comparing conventional aerobic counts with ROS-neutralised counts proved the latter to be beneficial for the enumeration of sub-lethally injured faecal indicator bacteria in water. In contrast to such results for bacteria exposed to brass, sunlight, high temperature and free chlorine, starvation or low pH conditions (Chapter 4) resulted in less evidence of such injury in *E. coli* and *Enterococcus faecalis* on selective or non selective media and the counts were not substantially affected by the growth conditions or growth media used for enumeration. The investigations satisfied the overall research aims of the present study and provided new information in advancement in enumerating sub-lethally injured bacteria under ROS- neutralised conditions.

The information gained from the investigations outlined above were firstly used to develop a novel broth-based assay for faecal coliforms and/or *E. coli* (Chapter 6) and then to compare this in an MPN multiwell format against an agar-based medium under laboratory conditions. Secondly evaluation was carried out for such a medium in a field-based-broth format (Chapter 7), in comparison to the currently available

field test method, (H_2S broth) and alongside a conventional plating method, including the operation of the medium with unskilled personnel in rural locations in India as a preliminary field trial of this approach. It was noted in the present study that the peroxide-neutralised conditions, created in the medium by incorporating 0.05% w/v sodium pyruvate, generally recovered the bacterial cells somewhat less than ROS-neutralised conditions but more than under aerobic and anaerobic growth conditions. The broth-based medium unlike an agar-based medium contains oxygen in much lower concentrations (typically at 5-10 ppm, rather than ≈ 20% in air) and peroxide-neutralised conditions might be sufficient to removing most of the oxygen toxicity created in a liquid growth medium. Anaerobic incubation (e.g. in a jar or cabinet) is also impractical under field conditions. The broth-based medium was developed so that it could enumerate sub-lethally injured bacteria and consisted of a selective agent (Tergitol 7), a peroxide-neutraliser (sodium pyruvate) in addition to a diagnostic agent (8-hydroxyquinoline-β-glucuronide) incorporated in the basal medium (Chapters 6 and 7). The development and evaluation of the novel medium (Coliblack) indicated it to be effective where the presence of *Escherichia coli* could easily be interpreted by field workers (Chapter 7) since it gave clear-cut colour change. A person without any scientific knowledge could use the Coliblack medium, which can also be stored for several months unlimited periods unlike many other growth media. In this respect, the original aims of the study have been fully addressed and the work represents advancement in an understanding of how to maximise enumeration of faecal indicator bacteria in water. Thus the development of the Coliblack medium proved to be an important achievement of one of the major research aims in the present study.

The concept of the viable but non-culturable (VBNC) cell has been controversial from its original proposal. Some authors have suggested it to be a differentiation process similar to spore formation (Roszak and Colwell, 1987) for example, different protein expression patterns have been reported for *Enterococcus faecalis* in the stationary state and the VBNC state (Heim *et al.*, 2002). Other researchers think it to be a survival strategy adopted by bacteria under harsh environmental conditions (Bar *et al.*, 2002; Asakura *et al.*, 2002; Mukamolova *et al.*, 2003; Aertsen and Michiels, 2004), while some explain it simply as a reversible state that consists of cells that can be cultured only under the most favourable growth conditions, for example by the addition of 0.05% of sodium pyruvate to the enumeration media in the case of *Vibrio vulnificus* (Bogosian *et al.*, 1998; Bogosian and Bourneuf, 2001), or by resuscitation of *Vibrio vulnificus* by nutrient addition (Whitesides and Oliver, 1997). The results from the present study put a question mark over VBNC hypothesis whose results are based on conventional aerobic counts, as debilitated bacterial cells that may not be cultured under such aerobic conditions may be culturable under ROS-neutralised conditions. Thus the VBNC hypothesis needs to be investigated further in this respect (see future work).

8.2 Future Research

The present study has investigated several stressful environmental factors that may cause injury to bacteria and has established resuscitation steps and media that can help to detect and enumerate primary faecal indicator bacteria, i.e. *Escherichia coli* (Clesceri *et al*,. 1998) and secondary faecal indicator bacteria i.e. *Enterococcus faecalis* (Anon., 2002) in environmental samples, particularly drinking water. Additional research could be carried out by extending the range of micro-organisms

studied taking into consideration other food-borne and water-borne bacteria as follows:

1) The VBNC theory could be investigated further using different methods such as traditional culture-based methods using ROS-neutralised medium compared with culture-independent methods such as fluorescence in situ hybridization (FISH) using specific oligonucleotide probes and direct viable counts (DVC), to examine the results obtained by each method (Bjergbaek and Roslev, 2005). Such studies would be a logical extension of the work of Bogosian *et al.* (2001) on *Vibrio vulnificus* to faecal indicator bacteria.

2) The practical use of copper and brass products could be studied in water and food microbiology as follows:

- Assessing the inactivation of specific pathogens causing water-borne diseases such as cholera (*Vibrio cholerae*), dysentery (*Shigella* spp.), typhoid (*Salmonella typhi*) and diarrhoea/gastro-enteritis (*Campylobacter* spp., enteropathogenic *E. coli*) as well as viral pathogens (e.g. rotavirus) in water stored in brass and copper vessels. Investigations could be carried out to study the effects of water quality including physical and chemical parameters such as turbidity, temperature, pH, amount of dissolved oxygen, soluble inorganic compounds and hardness of water, on the antimicrobial properties and availability of leached copper and zinc from brass and copper vessels.

- Evaluation of the application of copper utensils such as cups, jugs or bottles used to store drinking water. The use of copper products in reducing water-borne diseases could be investigated through field

studies including epidemiological work in rural villages in developing countries. The research could include the feedback and opinions/views of people in rural areas, in a similar way to that used to evaluate the novel field-based medium (Chapter 7).

- Investigation of the rate of bacterial injury and inactivation using oxidised (aged) and non-oxidised (clean metallic) surfaces of copper and brass, e.g. in the food industry using copper foils or metal surfaces for processing or preparing food items. Thus Faundez *et al.* (2004) discussed the antimicrobial activity of copper surfaces against suspensions of *Salmonella enterica* and *Campylobacter jejuni.* Similarly Beal *et al.* (2004) investigated the effect of copper ions on the death rate of *Salmonella typhimurium* DT104:30 prevalent in liquid food substrates including of liquid pig feed acidified by lactic acid, though the studies were confined to conventional aerobic enumeration procedures. Earlier observations that toxigenic *E. coli* may be killed by exposure to copper and brass surfaces of a few hours (Keevil *et al.*, 1999; Keevil, 2000; Keevil, 2001; Maule and Keevil, 2001) and a study showing that copper surfaces may be bactericidal for *E. faecalis* (Robine *et al.,* 2002) should be re-evaluated under ROS-neutralising conditions, since the plate count data were obtained in these studies only under standard aerobic conditions.

3) The development of a novel-broth field based medium could be extended as follows:

- Investigations could be made regarding the performance of other quenchers of ROS such as incorporation of the recovery enzyme Oxyrase® (Baylis *et al.*, 2000) into a broth-based medium. Oxyrase® is known to possess enzymes both to scavenge peroxides and to turn the medium anaerobic (Stephens *et al.*, 2000). Comparisons could be made for its performance with the peroxide-neutralised based broth medium described in Chapter 7. This aspect could be applied for the increased recovery of stressed bacteria prevalent in the food and water samples. However, oxyrase® could not be dried onto filter paper and would have to be added to the broth medium after autoclaving, thereby limiting its application in the format currently used for field medium (Chapter 7).

- Studies could focus on formulating a less inhibitory and less expensive growth medium containing an alternative glucuronide substrate as a diagnostic agent for the isolation of *Escherichia coli* in food and water samples. Preliminary studies were conducted to oxidize the inexpensive substrate aesculin a 6, 7-dihydroxycoumarin-β-glucoside in the presence of the enzyme glucose oxidase to produce the substrate aesculetin-glucuronic acid that might then be cleaved by β-glucuronidase to release aesculetin to react with a ferric salt to produce a non-diffusible dark brown/black-coloured complex but these trials were not successful. In the present study, during the early development of the novel broth-based medium (Chapter 6), two

different β-glucuronidase substrates were incorporated into the basal medium, namely MU-GLUC (which needs UV light for fluoroscence) and X-GLUC (which cannot be used under anaerobic conditions) and these were latter replaced by 8-HQ-GLUC, an expensive diagnostic agent (Chapter 7) costing £ 29 for 50 mg. Further research should aim at developing/incorporating a diagnostic agent which is much less expensive as it would be helpful in making the novel broth-based medium more cost-effective in rural locations in developing countries, where it would then have the potential for practical benefit.

Section 9

References

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Inactivation of *Escherichia coli* and coliform bacteria in traditional brass and earthernware water storage vessels

Puja Tandon[1], Sanjay Chhibber[2] and Robert H. Reed[1,*]
*[1]Division of Biomedical Sciences, Northumbria University, Ellison Place, Newcastle upon Tyne, Tyne and Wear NE1 8ST, UK; [2]Department of Microbiology, Panjab University, Chandigarh, India; *Author for correspondence (e-mail: rob.reed@northumbria.ac.uk; phone: +44-191-2274071; fax: +44-191-2273519)*

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Key words: Heavy metals, Reactive oxygen species (ROS), Selective media, Sub-lethal stress

Abstract

The detection and enumeration of indicator bacteria such as *Escherichia coli* is used to assess the extent of faecal contamination of drinking water. On the basis of this approach, the effectiveness of storing water contaminated with faecal indicator bacteria in brass or earthern vessels (mutkas) of the type used in rural India have been investigated. Suspensions of bacteria in sterile distilled water were maintained for up to 48 h in each vessel and enumerated by surface plate counts on nutrient agar (non-selective) and several selective coliform media at 37 °C either under standard aerobic conditions, or under conditions designed to neutralise reactive oxygen species (ROS), e.g. using an anaerobic cabinet to prepare plates of pre-reduced growth medium or by inclusion of sodium pyruvate in the growth medium, with incubation of aerobically-prepared plates in an anaerobic jar. The counts obtained for *E. coli* decreased on short-term storage in a brass mutka; counts for selective media were lower than for equivalent counts for non-selective medium, with ROS-neutralised conditions giving consistently higher counts than aerobic incubation. However, after 48 h, no bacteria were cultivable under any conditions. Similar results were obtained using water from environmental sources in the Panjab, and from rural households where brass and earthern mutkas are used for storage of drinking water, with enumeration on selective coliform media (presumptive total coliforms). In all cases results indicated that, while storage of water in a brass mutka can inactivate *E. coli* and coliforms over a 48 h period, standard aerobic plate counting using selective media may not be fully effective in enumerating sub-lethally damaged bacteria.

Abbreviations: mFC-R – membrane faecal coliform agar without rosolic acid; mLSA – membrane lauryl sulphate agar; ROS – reactive oxygen species

Introduction

Water-borne disease is a significant global issue; the consumption of water contaminated with faecal pathogens is one of the cause of up to 2.5 million deaths annually of children due to diarrhoea (Kosek et al. 2003). Despite major efforts to deliver safe, piped, community water to the world's population over the past two decades, the reality is that such supplies will not be available to all people for the forseeable future (Mintz et al. 2001).

Recent studies of practical interventions to create safe water have clearly demonstrated that

improved quality of water at the family level can have a significant impact in reducing water-borne diarrhoeal diseases (e.g. Wright et al. 2004). Many of the small-scale methods to purify water and make it safe for drinking and other household purposes can be traced back to ancient times; for example, Indian literature from 4000 years ago directed people to treat foul water either by exposure to sunlight (Reed 2004), by filtering through charcoal, or by storage in metal vessels made of copper, brass or silver. In several parts of rural India, including Rajasthan and Panjab, people continue to use brass or copper vessels (mutkas) to carry and store water and there is a general belief among the people that such metal containers have beneficial properties against the agents of water-borne disease (WHO 2002a).

Since the earliest microbiological investigations of drinking water quality, the detection of faecal indicator bacteria in drinking water has been used as a means of predicting the possible presence of pathogenic bacteria (WHO 2002b, 2004). This approach has also been used to investigate the microbiological quality of water samples taken from traditional water storage vessels (e.g. Khairy et al. 1982; Crump et al. 2004; Wright et al. 2004). However, these indicator bacteria may undergo variable amounts of growth inhibition, stress or injury in environmental waters (Clesceri et al. 1998). Sub-lethal physiological damage results from exposure to chemical, physical and/or biological factors and may cause the organisms to lose the ability to grow on those routine selective media that are otherwise satisfactory for the cultivation of healthy cells (Calabrese and Bissonnette 1990a; Kang and Siragusa 1999).

Copper and its alloys such as brass (2:1 w/w copper:zinc) exert their antimicrobial effect by interference with essential biological systems e.g. by altering the conformation of enzymes and other biomolecules, leading to cell death (Hassen et al. 1998). Toxicity is thought to result from the solubilisation of small amounts of metal ions from surfaces and this has been termed the 'oligodynamic' effect by Nageli (1893) to emphasise that it occurs at extremely low metal concentrations. Conventional toxicity testing of bacteria usually involves laboratory cultivation, either in an agar-based medium or in broth following exposure to the heavy metal, typically under standard aerobic conditions. However, recent studies on heat-stressed bacteria (Mizunoe et al. 1999; Stephens et al. 2000) have demonstrated that reactive oxygen species (ROS), derived mainly from aerobic respiration, may inactivate sub-lethally damaged bacteria and thereby preventing their enumeration. The so-called 'suicide hypothesis' (Aldsworth et al. 1999) further develops this concept, proposing that growth-arrested bacterial cells with damaged antioxidant systems can undergo a process of self-destruction when cultured under aerobic conditions, where respiratory metabolism produces a burst of intracellular ROS that is uncoupled from growth.

The present study was carried out to investigate the inactivation of *Escherichia coli* and coliform bacteria in traditional Indian brass and earthernware water storage vessels, with enumeration on selective and non-selective media to test for sub-lethal damage (McFeters et al. 1986). Aerobic counts were compared to ROS-neutralised counts, to establish the significance of respiration-induced self-destruction. Environmental water samples were also stored in similar containers and tested for total coliform bacteria, as were samples of drinking water collected directly from brass and earthern mutkas from households in rural Panjab, enumerated on different selective media under aerobic and ROS-neutralised conditions. The results demonstrated that lower counts were obtained for bacteria kept for 6–24 h in water stored in the brass mutka compared to the earthernware vessel, but that additional reductions in counts were caused by the use of certain selective media and by aerobic enumeration. Overall, the results indicate that the extent of short-term inactivation of bacteria in a brass storage vessel may be substantially overestimated using conventional selective media and aerobic culture methods.

Materials and methods

Bacterial cultures

E. coli strain NCTC8912 was obtained from the National Collection of Type Cultures, Colindale, UK and studied at the University of Northumbria, while strain TN675 from the Central Research Division, Osaka, Japan and isolates PUCC061 and PUCC113 (wild isolates from local natural waters) Panjab University Culture Collection, were studied at Panjab University, Chandigarh. Stocks were

maintained by repeated sub-culture on nutrient agar (Oxoid, Basingstoke, UK) at 37 °C. Standard experimental cultures were prepared by loop inoculation of bacteria into nutrient broth (Oxoid), followed by overnight incubation at 37 °C, without shaking, where the bacteria reach stationary phase in a fermentative mode, with no detectable oxygen in the broth on harvest (Khaengraeng and Reed in press). All experiments were performed twice for each of the isolates and strains of *E. coli.*

Water sources

Laboratory experiments used sterile distilled water, adjusted to pH 7.0. Environmental water samples were taken from three rivers in Panjab using sterile containers. The experiments were carried out twice for each river, with triplicate counts by membrane filtration in each instance. All samples were collected between 8:00 am and 9:00 am to avoid overexposure to sunlight, and taken in darkness to the Microbiology Department of Panjab University, Chandigarh within 90 min, for processing. Field samples were taken from brass and earthen mutkas used for drinking water storage within individual households in three Panjabi villages: (i) the hand pump in Gujrawala; (ii) the municipal committee tap in Doraha; and (iii) the municipal committee tap of Sector 40 in Chandigarh.

Storage vessels

Brass and earthern mutkas (12 l capacity) were obtained from local merchants in Doraha. Before use, they were scrubbed thoroughly with a non-abrasive cloth to remove any adherent microflora/biofilm, disinfected using 0.1% (w/v) Virkon, rinsed three times with sterile distilled water, then soaked for at least 3 h and re-rinsed three times, to remove all traces of disinfectant. In some experiments, stainless steel, copper and glass vessels of equivalent volumes were also tested.

Preparation of media

All media were obtained commercially, including nutrient agar and MacConkey agar (Oxoid, Basingstoke,UK), plus *m*-lauryl sulphate agar (mLSA) medium, prepared by the addition of 1.5% w/v bacteriological agar (Oxoid) to *m*-lauryl sulphate broth (Merck, Darmstadt, Germany). These media were sterilised by autoclave (121 °C, 15 min), while mFC agar without rosolic acid (mFC-R, Merck) and mEndo agar (Merck) were boiled. Plates were prepared as described by Clesceri et al. (1998) in sufficient quantities to allow counts to be performed in triplicate, with and without added 0.05% w/v sodium pyruvate as a specific neutralising agent for peroxides (Khaengraeng and Reed in press). Plates were pre-dried in a sterile laminar flow cabinet (20 min) before use.

Preparation of cell suspensions

Overnight broth cultures of *E. coli* were centrifuged at 5300 × *g* for 5 min at 5 °C, washed twice with quarter-strength Ringer's solution to remove any traces of the growth medium and then suspended in sterile distilled water, with a pH adjusted to 7, to give a final dilution of 1:100, i.e. 160 ml broth to 16 l of suspension.

Laboratory experiments

The above cell suspension was poured equally (8 l) into (i) the brass mutka and (ii) the earthernware mutka, and kept at 25 °C. Samples were taken at 0, 6, 24, and 48 h and processed by serial decimal dilution in quarter-strength Ringer's solution (pH 7.4) to cover the dilution range 10^0–10^{-4}. Surface spread plates were prepared using 0.02–1.0 ml of each dilution. Plates were incubated at 37 °C for 24 h under aerobic conditions, or 48 h under anaerobic conditions, obtained either (i) using an anaerobic jar (Merck Anaerocult®; Anon 2000) or (ii) using an anaerobic cabinet (Biotrace Fred Baker, Bridgend, Wales) for UK experiments. After incubation, the plates were examined for colonies, expressed as CFU ml^{-1} by correcting for dilution and volume and further incubated aerobically for up to 72 h to confirm that no additional colonies had grown, before being discarded. All plate counts were performed in triplicate, and geometric means and 95% confidence limits were first calculated following log-transformation to normalise the data (Anon 2002) and then

converted back to non-log numerical format for ease of comparison. Comparisons between two different mean values can be based on overlap or non-overlap of their respective 95% confidence limits – thus, two means with non-overlapping 95% confidence limits can be regarded as different, whereas two means with overlapping confidence limits cannot.

Environmental water samples

Having established from the initial studies with pure cultures of *E. coli* that ROS-neutralised conditions could be obtained using a combination of a growth medium with added sodium pyruvate coupled with incubation in an anaerobic jar, aliquots of the natural waters were processed by standard bacteriological membrane filtration using 1.0–100.0 ml of water filtered through 47 mm diameter membranes of 0.45 μm pore size (Pall-Gelman, Ann Arbor, USA) and then enumerated on all of the selective media (MacConkey, mFC-R, mEndo and mLSA) either (i) aerobically for media without added pyruvate (standard aerobic conditions) or (ii) in an anaerobic jar for media with 0.05% sodium pyruvate (ROS-neutralised conditions) to provide initial counts, prior to storage. Water (8 l) from each of the sources was poured into the brass and earthern mutkas and incubated at 28 °C, with sampling and enumeration by membrane filtration. The number of presumptive coliforms (total coliforms) in each sample was calculated per 100 ml by multiplying the colony count (CFU) by 100 and dividing by the sample volume in ml.

Household water samples

A total of 12 different households were selected for testing. Samples were collected between 8:00 am and 9:00 am from brass and earthern mutkas in use by individual households in rural locations in Panjab, where the water had been collected the previous day and stored overnight (12–15 h). These samples, along with water obtained at the same time from each of the sources used to fill these vessels (and stored overnight in freezer prior to testing), were returned to the laboratory within 90 min and processed by membrane filtration using the selective media described above, to provide an indication of the initial level of total coliforms in the water to compare with the values obtained for the stored water. Preliminary experiments showed that overnight storage in a refrigerator had no substantial effect on the counts of the source water.

Results

Effects of enumeration conditions on colony counts of E. coli suspended in water maintained in brass and earthern storage vessels

Plate counts are shown in Figure 1a for a suspension of stationary phase *E. coli* NCTC8912 prior to storage (0 h), enumerated on nutrient agar in the presence and absence of 0.05% w/v sodium pyruvate either aerobically, or under one of three different sets of anaerobic condition. In the first two sets of anaerobic conditions, dilution and spread plating were carried out on the laboratory bench under aerobic conditions and the plates were then transferred either to an anaerobic jar, which took around 1 h to create fully anaerobic conditions (Anon 2000), or to an anaerobic cabinet, which provided a fully anaerobic atmosphere from the start. In the third set, dilutions were performed and plates were prepared entirely within the anaerobic cabinet, with spread plating onto pre-reduced medium (kept for 24 h in the anaerobic cabinet beforehand), with incubation in the same cabinet to provide anaerobic conditions throughout. The results indicate that at 0 h there were no substantial differences between the counts obtained under aerobic conditions, or in any of the anaerobic systems irrespective of whether the medium contained added pyruvate or not (Figure 1a). In contrast, following 6 h incubation in the brass mutka, there were substantial decreases in counts compared to the initial values (Figure 1b), with the reduction varying from less than 10-fold to over 1000-fold, depending upon the enumeration conditions. Water stored in the brass vessel and then enumerated under aerobic conditions in pyruvate-unsupplemented medium showed the lowest count, with pyruvate-supplemented plates giving a 70-fold higher count than for unsupplemented medium under aerobic conditions. A similar pattern of higher counts for

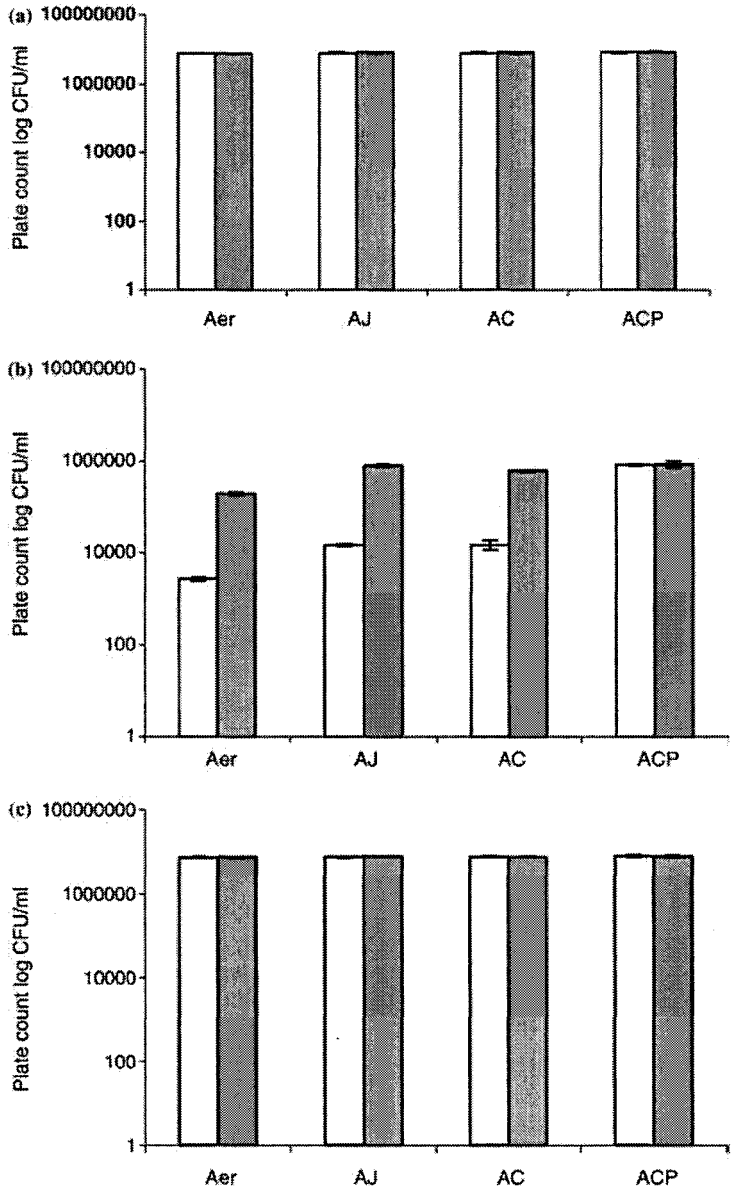

Figure 1. Effects of storage on counts of a suspension of *Escherichia coli* NCTC 8912. (a) at 0 h in sterile distilled water; (b) following 6 h storage in a brass mutka; (c) following 6 h storage in an earthern mutka, enumerated on nutrient agar (unshaded bars) or on nutrient agar supplemented with 0.05% w/v sodium pyruvate (shaded bars) prepared and cultured under aerobic conditions (Aer), prepared under anaerobic condition and cultured either in an anaerobic jar (AJ) or in an anaerobic cabinet (AC), or prepared under anaerobic conditions using pre-reduced medium and maintained in an anaerobic cabinet (ACP). Error bars represent 95% confidence limits (n = 3).

pyruvate-supplemented medium was also observed for plates prepared in air and then transferred either to the anaerobic jar or to the anaerobic cabinet. In contrast, no such difference in counts was observed for pyruvate-supplemented and unsupplemented plates prepared and maintained in the anaerobic cabinet using pre-reduced media (Figure 1b). Thus a count of approximately 10^6 CFU

ml^{-1} was obtained for four sets of conditions, namely samples processed under aerobic conditions on pyruvate-supplemented medium which was then incubated either (i) in an anaerobic jar or (ii) an anaerobic cabinet, along with samples processed within the anaerobic cabinet and plated onto either pre-reduced (iii) pyruvate-supplemented or (iv) unsupplemented growth medium. These results indicate that the combination of an anaerobic jar and a pyruvate-supplemented medium can give a count equivalent to that obtained when using pre-reduced unsupplemented medium in an anaerobic cabinet. Presumably, in all of these four sets of conditions, the damaging effects of ROS are neutralised. In subsequent experiments in India, ROS-neutralised conditions were obtained by carrying out dilution and plating in air using pyruvate-supplemented media with subsequent incubation in an anaerobic jar, as an anaerobic cabinet was not available. After 24 h incubation in the brass mutka, the stored water gave no detectable counts for any growth medium or incubation conditions.

Figure 1c shows equivalent data for the same suspension kept in the earthern mutka for 6 h. In contrast to the results for brass, storage in earthernware resulted in no substantial change in count for any of the conditions used. Similarly, after 24 h incubation in the earthernware mutka, there was minimal change in the count across the various growth conditions (data not shown).

Experiments were also performed using a copper and stainless steel mutka, and a glass flask of equivalent volume, for comparison with the results for brass and earthernware. *E. coli* suspensions stored in the copper vessel showed a similar overall pattern in counts after 6 h to that seen with the brass vessel while the stainless steel mutka and glass flask showed similar minimal changes to those of the earthern mutka for counts up to 48 h (data not shown). Additional experiments were carried out using quarter-strength Ringer's solution (pH 7) for comparison with sterile distilled water showing similar overall results (data not shown) and indicating that the presence of small amounts of dissolved inorganic salts in the suspension medium has no substantial effect on the overall pattern of inactivation.

Measurements of dissolved copper and zinc were performed by atomic absorption spectrophotometry following storage of water for 48 h in the brass mutka, giving values below 0.1 ppm in both instances. Even by consuming 10 l of such water in a single day, a person would be well within the daily recommended maximum intake for both metals (Domek et al. 1984; Grey and Steck 2001).

Counts are shown in Figure 2a for a suspension of *E. coli* NCTC8912 at 0 h, plated onto either nutrient agar (non-selective medium), MacConkey agar, mFC-R, mEndo agar or mLSA (selective media) and enumerated either (i) aerobically (unsupplemented medium) or (ii) under ROS-neutralised conditions (pyruvate-supplemented medium incubated in an anaerobic jar). Similar counts were obtained for all media, irrespective of the enumeration conditions. A similar pattern (data not shown) was also obtained after 24 h suspension of *E. coli* NCTC8912 in an earthernware vessel. In all subsequent experiments using other *E. coli* strains a similar pattern was observed for the initial inoculum and for cells kept in the earthern mutka (data not shown).

Figure 2b shows *E. coli* NCTC8912 counts following 6 h incubation in the brass mutka. The overall results showed 20–20000-fold lower counts when compared to the initial inoculum, with the extent of the decrease showing a strong dependence on both the medium and the growth conditions used. Comparing the selective coliform media incubated under aerobic conditions, MacConkey agar gave a similar count to nutrient agar, mLSA gave the lowest aerobic count and mFC-R together with mEndo medium gave intermediate values. Under ROS-neutralised conditions, nutrient agar showed an increase in colony count of around 20-fold whereas the selective media gave more variable increases, with none of them achieving the ROS-neutralised count of that seen with nutrient agar. However, the same trend was noted under ROS-neutralised conditions as was observed for aerobic conditions, with MacConkey agar giving a count closest to that of nutrient agar and mLSA giving the lowest overall count. Further incubation of all media for up to 72 h gave no substantial increase in colony counts under any conditions, confirming that the initial incubation period was sufficient to give colonies of a size large enough to be counted.

Figure 3a shows a similar experiment carried out using stationary phase *E. coli* TN675 suspended for 6 h in the brass mutka, while Figure 3b

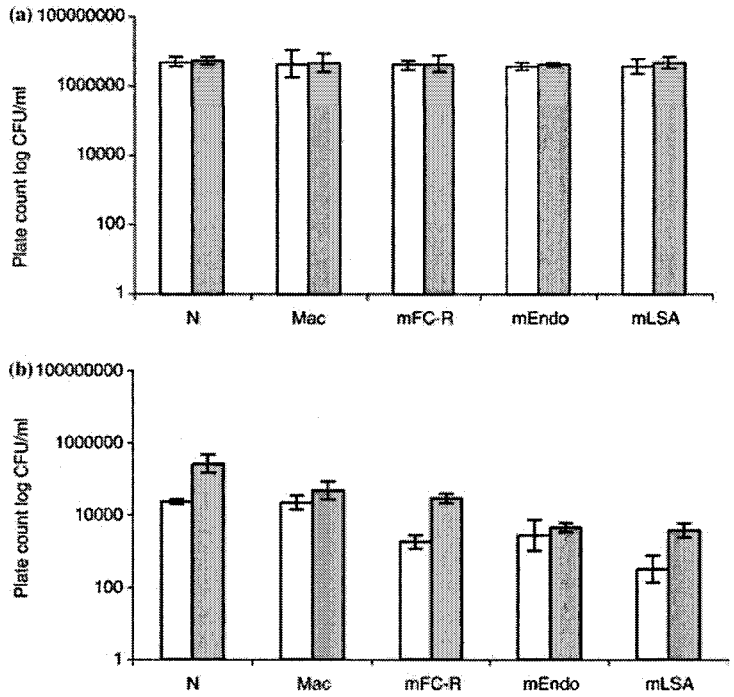

Figure 2. Effects of storage on counts of suspensions of *Escherichia coli* NCTC 8912 using non-selective and selective growth media. (a) *E. coli* NCTC8912 at 0 h in sterile distilled water (b) *E. coli* NCTC8912 after 6 h in a brass mutka; enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), *m*-lauryl sulphate agar (mLSA), mEndo medium, and *m*-FC agar without rosolic acid (mFC-R), incubated either aerobically (unshaded bars) or anaerobically with supplementation of 0.05% w/v sodium pyruvate in an anaerobic jar (shaded bars). Error bars represent 95% confidence limits (n = 3).

and c shows equivalent results for the two Indian isolates, *E. coli* PUCC061 and PUCC113, suspended for 24 h in the brass mutka (after 6 h there was minimal change in the counts of the environmental isolates). Overall, the broad trends shows in Figure 3a–c are similar to those observed with *E. coli* NCTC8912 (Figure 2b), confirming that non-selective nutrient agar under ROS-neutralised conditions gave substantially higher counts than under aerobic conditions, and compared to all of the selective media, with MacConkey agar proving least inhibitory and mLSA most inhibitory in all cases. However, a reduced effect of ROS neutralisation was seen with the two environmental isolates, compared with the laboratory strains, which indicates that they may be somewhat less susceptible to respiratory self-destruction. This is also consistent with their lower overall rate of inactivation in the brass mutka, requiring 24 h to give broadly similar decreases in plate counts to those observed after 6 h for *E. coli* NCTC8912 (Figure 2b) and TN675 (Figure 3a). However, for all four tested *E. coli* strains, suspensions of cells kept in water for 48 h in the brass mutka gave no detectable counts on nutrient agar or on any selective medium, whether incubated under aerobic or ROS-neutralised conditions, confirming the inhibitory effects of storage in the brass vessel.

Effects of enumeration conditions on coliform counts for environmental water samples kept in brass and earthern storage vessels

Figure 4a, c and e shows result obtained for presumptive total coliform counts obtained by membrane filtration of three environmental water samples, from Khamanon, Doraha and Khanpur rivers, using four different selective media under aerobic and ROS-neutralised conditions. A broadly similar initial count was obtained for each water sample, irrespective of the selective medium

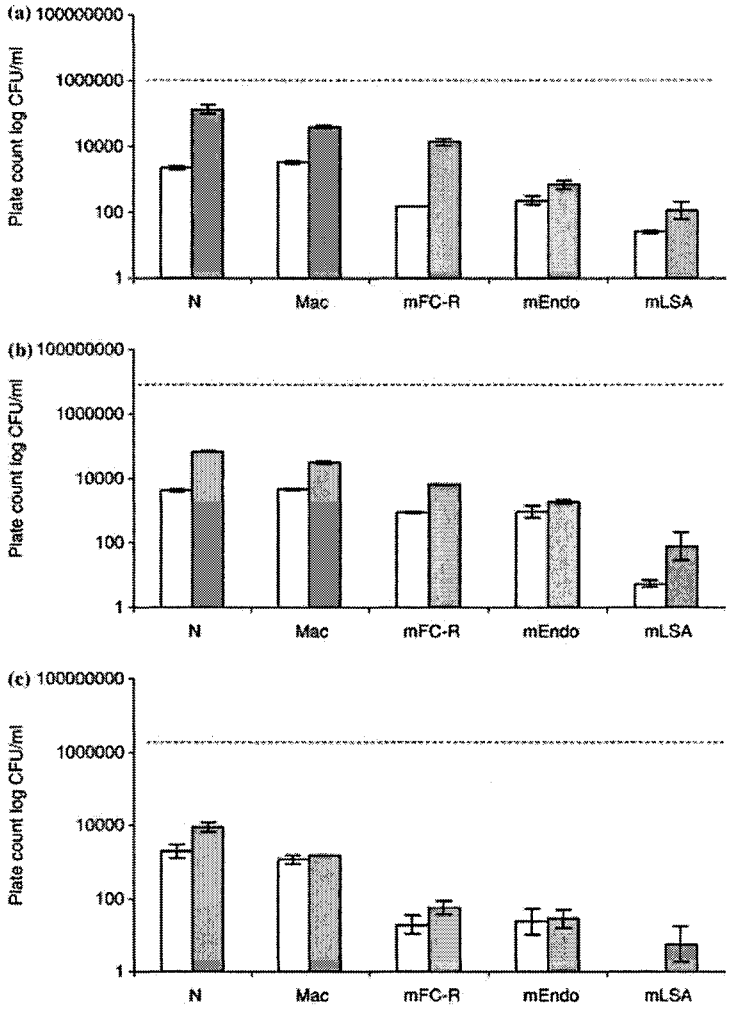

Figure 3. Effects of storage on counts of suspensions of three *Escherichia coli* isolates. (a) *E. coli* TN675 after 6 h in a brass mutka; (b) *E. coli* PUCC061 after 24 h in a brass mutka; (c) *E. coli* PUCC113 after 24 h in a brass mutka, enumerated on non-selective nutrient agar (N), MacConkey agar (Mac), *m*-lauryl sulphate agar (mLSA), mEndo medium, and *m*-FC agar without rosolic acid (mFC-R), incubated either aerobically (unshaded bars) or anaerobically with supplementation of 0.05% w/v sodium pyruvate in an anaerobic jar (shaded bars). The initial inoculum is represented by the dotted line in each case. Error bars represent 95% confidence limits ($n = 3$).

used, or whether the plates were incubated aerobically on unsupplemented medium or anaerobically on pyruvate-supplemented medium, though there was some indication of a slightly higher count on MacConkey agar and a slightly lower value on mLSA for Khanpur river water.

Figure 4b, d and f shows the presumptive total coliform counts obtained when samples of the same three river waters were suspended in the brass mutka for 6 h. Overall, the coliform counts decreased by around 100-fold on MacConkey agar, with greater reductions seen for the other three selective media, and especially for mLSA, which gave no detectable count under aerobic conditions in one of the three samples (Figure 3b). As with the pure cultures of *E. coli*, enumeration under ROS-neutralised conditions gave higher counts than conventional aerobic incubation, though the increases observed with the environmental water samples were somewhat less than those seen with the pure cultures (cf. Figure 4 and Figures 2–3). After 48 h storage within the brass

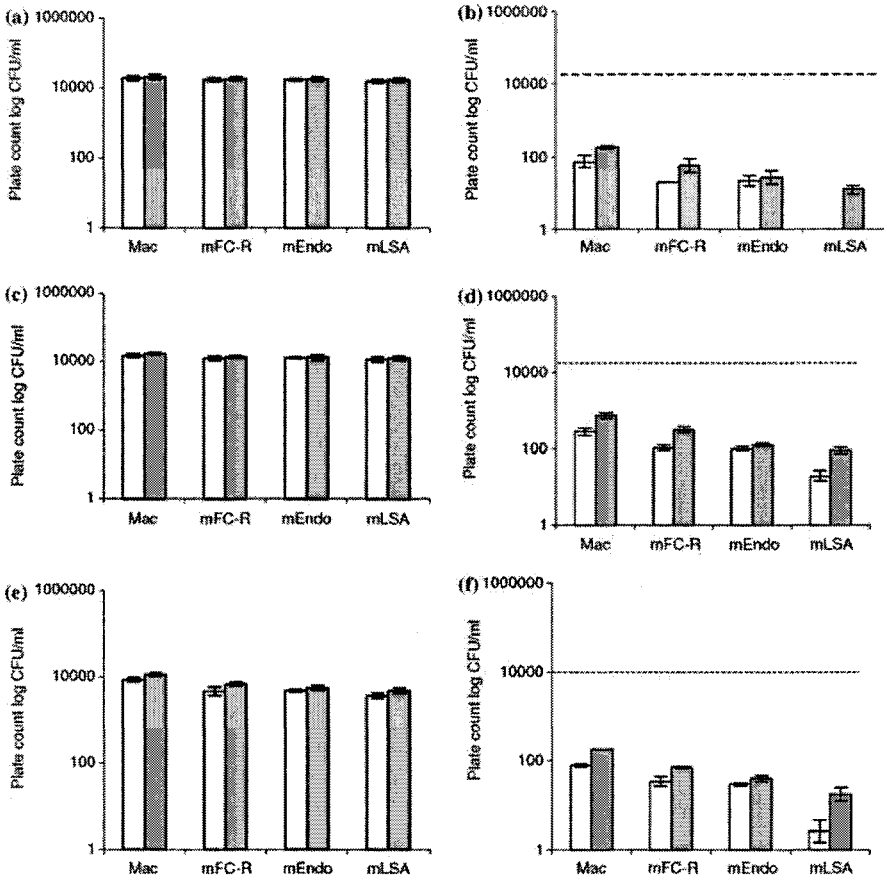

Figure 4. Effects of storage on total coliform counts of environmental water samples. (a) Khamanon river water at 0 h; (b) Khamanon river water after 6 h in a brass mutka; (c) Doraha river water at 0 h; (d) Doraha river water after 6 h in a brass mutka; (e) Khanpur river water at 0 h; (f) Khanpur river water after 6 h in a brass mutka, enumerated using MacConkey Agar (Mac), *m*-lauryl sulphate agar (mLSA), mEndo medium, *m*-FC agar without rosolic acid (mFC-R), incubated either aerobically (unshaded bars) or anaerobically with supplementation of 0.05% w/v sodium pyruvate in an anaerobic jar (shaded bars). The initial inoculum is represented by the dotted line. Error bars represent 95% confidence limits (n = 3).

mutka all three-river waters gave no detectable count on any medium, whether incubated under aerobic or ROS-neutralised conditions, confirming the inhibitory effects of brass.

Effects of enumeration conditions on water samples taken from brass and earthern mutkas in rural Panjab households

Table 1 shows data for coliform counts obtained by membrane filtration of water samples collected from three representative sets of household mutkas following overnight storage (12–15 h) and tested on two occasions, alongside counts for the source water used to fill the storage vessels. Similar overall results were also obtained for the other households (data not shown). The mean presumptive total coliform count was obtained for the four tested selective media, both aerobically and under ROS-neutralised conditions, as in previous experiments. The source waters showed some evidence of different counts with the various media used, with the lowest values for mLSA under aerobic conditions. Storage of water overnight in an earthern vessel resulted in a decrease in aerobic count of between 15 and 78%, with MacConkey agar giving the smallest decreases and mLSA the

Table 1. Coliform counts for source and overnight stored water from households in three rural locations in Panjab.

Source sampling date	Sample type	MacConkey agar		mFC agar-rosolic acid		*m*-Endo agar	
		$+O_2$	$-O_2$ + P	$+O_2$	$-O_2$ + P	$+O_2$	$-O_2$ + P
Chandigarh 20.2.03	S	7032 (6407–7718)	7564 (6552–8731)	6230 (5391–7201)	6766 (6289–7278)	6196 (5166–7615)	6665 (5832–7615)
	E	5933 (5690–6185)	6766 (6137–7457)	2932 (2470–3479)	3864 (3257–4589)	2461 (1722–3517)	3665 (3205–4192)
	B	98 (82–117)	183 (160–210)	35 (27–45)	57 (53–63)	16 (12–21)	21 (16–26)
Doraha 21.2.03	S	6929 (5725–8386)	7497 (6433–8737)	6432 (5810–7121)	6766 (6137–7458)	6129 (5142–7308)	6399 (5984–6844)
	E	5899 (5485–6346)	6696 (5522–8118)	3728 (2794–4974)	4660 (3525–6160)	2363 (1785–3129)	2864 (2273–3609)
	B	383 (302–485)	590 (485–717)	275 (164–462)	343 (284–415)	256 (167–392)	316 (257–390)
Gujrawala 27.2.03	S	1417 (1352–1484)	1773 (1668–1885)	950 (843–1070)	1146 (967–1358)	816 (660–1008)	973 (845–1120)
	E	1270 (1187–1359)	1677 (1612–1744)	886 (757–1037)	1003 (979–1028)	666 (548–810)	860 (778–950)
	B	53 (38–73)	131 (120–142)	28 (25–30)	66 (51–86)	24 (20–30)	36 (25–51)
Doraha 25.3.03	S	8098 (7282–9006)	9796 (8427–11387)	7296 (6113–8707)	8297 (7242–9507)	6896 (5719–8314)	7966 (7487–8476)
	E	5732 (5114–6425)	7832 (7204–8515)	3965 (3356–4684)	4621 (3160–6758)	2854 (1734–4696)	3700 (3309–4291)
	B	386 (268–556)	1079 (872–1336)	180 (180)	357 (311–409)	93 (53–162)	255 (130–498)
Gujrawala 25.3.03	S	9830 (8655–11166)	11996 (10774–13359)	6996 (5818–8412)	8566 (8086–9075)	5561 (4400–7029)	6925 (5379–8917)
	E	8129 (6870–9619)	10428 (8820–12329)	5498 (4701–6428)	6629 (5429–8093)	3593 (2603–4960)	4729 (3766–5938)
	B	706 (546–913)	1343 (1179–1529)	292 (185–462)	633 (534–751)	73 (53–102)	200 (200)
Chandigarh 15.5.03	S	14231 (12972–15612)	17298 (16055–18638)	12999 (12167–13888)	15899 (15062–16783)	12898 (11670–14254)	13600 (13176–14063)
	E	9798 (8717–11012)	12566 (12081–13071)	6360 (5011–8072)	8099 (7391–8874)	6400 (5984–6844)	6964 (6030–8042)
	B	303 (280–329)	760 (689–838)	33 (24–46)	77 (70–84)	33 (22–50)	55 (53–58)

Mean count (upper and lower 95% confidence limits shown in brackets below each mean value) for presumptive total coliforms isolated from each water sample using a range of selective media, incubated either aerobically ($+O_2$) or anaerobically with supplementation of 0.05% w/v sodium pyruvate in an anaerobic jar ($-O_2$ + P). S: source water (stored overnight in refrigerator). E: water from earthernware mutka (12–15 h storage, ambient temperature). B: water from brass mutka (12–15 h storage, ambient temperature). Samples noted as <1, gave no detectable coliforms in triplicate 100 ml samples.

greatest proportional reductions. Under ROS-neutralised conditions, the counts for water stored in the earthern mutkas were somewhat higher, but the same overall trend was observed for the relative performance of the selective media. Overall, the results indicate that while storage overnight in earthernware may cause some sub-lethal damage, there is less evidence of irreversible inactivation, since the ROS-neutralised counts on MacConkey agar were mostly >80% of the corresponding values for the source water.

Table 1 also shows the data for brass mutkas, where a far greater effect of overnight storage was observed than was seen with earthern mutkas. The counts obtained differed, depending upon which selective medium was used, in the general sequence (from highest to lowest count): MacConkey > mFC-R > mEndo > mLSA. The aerobic MacConkey count was typically around 4% of that obtained for the source water, with even lower values for the other selective media: for example, in three of the six brass mutkas listed in Table 1, no colony count was obtained for 100 ml of water enumerated aerobically on mLSA. The ROS-neutralised counts for water from the brass mutkas were often around two or three times higher than those of the corresponding aerobic counts.

Taken together with the results for the pure cultures and the environmental water samples, the results in Table 1 indicate that storage in a brass vessel can cause both (i) sub-lethal damage, as demonstrated by the reduced counts under aerobic conditions and on more inhibitory selective media such as mLSA, and (ii) irreversible inactivation, as evidenced by the counts under ROS-neutralised conditions on less inhibitory medium such as MacConkey agar.

Discussion

The inhibitory effects of heavy metals such as copper, zinc and silver on aquatic micro-organisms are well established (e.g. Jonas 1989) and their biocidal properties has been exploited for control purposes, e.g. copper has been used against microbial biofilms (Kielemoes and Verstraete 2001), while the antimicrobial effects of silver have been rediscovered more recently in water treatment (Pedazhur et al. 1997). However, the influence of oxygen status on the enumeration of metal-stressed microbes does not appear to have been considered in earlier studies. In contrast, several researchers have studied the influence of oxygen and its by-products (ROS) on bacterial enumeration following exposure to other stressors. Thus, enhanced counts of *E. coli* after freezing or heat injury have been observed on addition of pyruvate-neutralising compounds such as catalase or pyruvate to agar media (e.g. Czechowitz et al. 1996). Similarly, chlorine-stressed coliforms have been shown to give enhanced counts on pyruvate-supplemented media, and this has been explained in terms of damage to cellular antioxidant defence systems, including catalase (Calabrese and Bissonnette 1990a). The benefits of adding pyruvate to coliform media have been noted on several occasions (e.g. Calabrese and Bissonnette 1990b; Sartory and Howard 1992) and this is usually ascribed to the quenching of peroxides generated by auto-oxidation of medium components (Sartory 1995). Beneficial effects of adding pyruvate were also observed in the present study, e.g. for *E. coli* cells suspended in water stored in a brass vessel (Figure 1b). However, a further enhancement in counts was obtained on incubation of cells plated onto pyruvate-supplemented medium under anaerobic conditions, indicating that while peroxides may be quantitatively the most important ROS, they are not the sole source of the growth inhibition observed under standard aerobic conditions.

The studies of George et al. (1998) on the effect of oxygen concentration on the recovery of sub-lethally heat-damaged bacteria support the concept that growth under anaerobic conditions can give higher counts than under aerobic conditions. These researchers observed that bacterial heat resistance was greater when enumerated under fully anaerobic conditions, whereas conventional aerobic enumeration suggested an apparent heat resistance that was far lower, with obvious implications for thermal processing of foodstuffs. Such findings are in agreement with the results obtained in Figures 1–4 and Table 1, where ROS-neutralised counts of bacteria kept in brass vessels were consistently higher than their aerobic counterparts.

The work of Stephens et al. (2000) points to two sources of oxidative stress during culture, namely from ROS due to (i) reactive components of the growth medium and (ii) cellular respiration, the

latter being responsible for the phenomenon of respiratory self-destruction of sub-lethally damaged cells under aerobic conditions (Aldsworth et al. 1999). It is possible to interpret the results of the present study primarily in terms of the respiratory self-destruction hypothesis; thus, the aerobic count of bacteria kept in the brass vessel and then enumerated on non-selective nutrient agar represents the number of cells whose antioxidant defence systems remain healthy enough to cope with growth in a fully oxygenated atmosphere, while the increase in counts observed under ROS-neutralised conditions, whether achieved either (i) by a combination of aerobic processing on a pyruvate-supplemented medium with subsequent transfer to an anaerobic jar, or (ii) by anaerobic processing and incubation on pre-reduced unsupplemented medium entirely under anaerobic conditions, represents that fraction of cells whose antioxidant defence systems are unable to cope in air. In the absence of any other stressors, the difference between the initial count and the ROS-neutralised count on such a non-selective medium may thus provide a measure of the number of cells whose metabolic processes have been damaged to the point where they are no longer able to replicate under aerobic conditions. Applying these principles to the data in Figure 1, the similar counts obtained under aerobic and ROS-neutralised conditions at the outset of the experiment shows that the cells were initially healthy (Figure 1a), and remained so on storage for 6 h in the earthern vessel (Figure 1c). In contrast, the number of healthy cells of *E. coli* NCTC8912 remaining after storage for 6 h in a brass mutka dropped to less than 0.1% of the initial value, while approximately 10% of the remaining fraction were sub-lethally damaged, with around 90% of the cells being irreversibly inactivated (Figure 1b). Since similar results were obtained with copper and brass, but not with earthernware, stainless steel or glass vessels, it would seem appropriate to conclude that these effects are due primarily to the dissolution of heavy metals into the water during storage.

The positive effects of added pyruvate on counts of *E. coli* exposed to brass and copper observed in the present study contrast with the observations of Grey and Steck (2001), who found no increase in plate counts of *E. coli* ED8739 following exposure to 0.5 mmol l^{-1} copper sulphate when the growth medium was supplemented with 0.32% w/v sodium pyruvate. While such differences may be explained in part by the use of different strains and experimental conditions, their evidence that such cells can be resuscitated by rinsing and incubation for several days in a copper-free 0.9% w/v NaCl solution is also consistent with the idea that metal-treated cells are sub-lethally damaged to the point where they are unable to grow on agar-based media under conventional aerobic conditions. Our results are also in agreement with their observation that '...current growth-based microbiological methods for assaying toxicity result in an under-count of the number of viable cells...' (Grey and Steck 2001), though our interpretation favours the respiratory self-destruction hypothesis, based predominantly on peroxide sensitivity (Bogosian et al. 2000), rather than the notion of a distinct and separate viable-but-non-culturable state.

Domek et al. (1984) performed experiments in which coliforms were exposed to dissolved copper and they reported that up to 90% of these bacteria showed sub-lethal damage, as demonstrated by their lack of growth on a selective medium, in contrast to an equivalent non-selective medium. Similar findings have also been obtained by other researchers, showing that coliform bacteria, including *E. coli*, typically fail to grow on selective media following sub-lethal injury or environmental stress (e.g. Bissonnette et al. 1974; Kang and Siragusa 1999). The results of the present study can be interpreted in a similar manner, with *E. coli* and coliforms exposed to copper and zinc for 6–24 h in a brass vessel showing decreased counts on certain selective media and especially in the presence of sodium lauryl sulphate (Figures 2 and 3 and Table 1). Given that most selective agents for coliforms are surfactants, such toxicity may be mediated through membrane damage, as evidenced by disruption to membrane-associated metabolic functions such as respiration (Domek et al. 1987) and solute transport (Sunda and Huntsman 1998), providing a plausible explanation for the reductions in counts on selective media observed in Figures 2–4.

Longer-term incubation of *E. coli* in the brass mutka led to cells being undetectable after 48 h even when enumerated under ROS-neutralised conditions, which indicates that prolonged storage in a brass vessel can fully inactivate the bacteria, in agreement with earlier reports (e.g. Patwardhan 1990). However, in practice, the villagers whose

mutkas were sampled in the present study do not routinely store their water for this period of time, but use the water 0–24 h of collection. Thus while Table 1 shows that overnight storage in a brass mutka can lead to a substantial reduction in coliform count, this time period was insufficient to completely inactivate these bacteria. Assuming that such results are also applicable to pathogenic bacteria, the implications are that storage for 12–15 h in a brass vessel does not necessarily render the water safe to drink. Furthermore, any post-collection contamination of the stored water (Wright et al. 2004) would add further to the microbial load, and thereby reduce the beneficial effects of storage in a brass or copper vessel. While anything that partially or fully inactivates bacteria has the potential to be beneficial under field conditions, we have also noted that villagers increasingly favour stainless steel or plastic mutkas, either because they are cheaper to purchase or because they are more durable than some traditional materials; however, such vessels would not be expected to show a significant reduction in bacterial counts over the storage period.

The results of the present study clearly demonstrate that many *E. coli* cells may be sub-lethally injured, rather than killed, by short-term incubation in a brass vessel (Figures 1–3). Such findings indicate that earlier observations that toxigenic *E. coli* may be killed by exposure to copper and brass surfaces of a few hours (Keevil et al. 1999; Keevil 2000) should be re-evaluated under ROS-neutralised conditions. The data for household mutkas in Table 1 shows how false-negative coliform counts might be obtained for particular combinations of selective medium and growth conditions; thus mLSA medium incubated aerobically gave no detectable coliforms from brass mutkas on several occasions when these bacteria were clearly detectable in large numbers using MacConkey medium under ROS-neutralised conditions. Such findings have obvious implications for the detection and enumeration of metal-stressed coliforms and *E. coli* in environmental water samples.

The present study indicates that for maximum enumeration of metal-stressed *E. coli* and coliforms, plate counts should be performed under ROS-neutralised conditions, either under fully anaerobic conditions with pre-reduced growth medium, or using a combination of an anaerobic jar with a pyruvate-supplemented medium. Such anaerobic effects should not be confused with the known enhancement of metal ion toxicity under anaerobic conditions, e.g. where cupric ions (Cu^{++}) are converted to the more toxic cuprous (Cu^{+}) form (McBrian 1980).

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